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ANALYSIS OF COPROSTANOL, AN INDICATOR OF FECAL CONTAMINATION

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Florida University

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16. Abstract

Gas chromatographic (GC) analysis of coprostanol was improved by showing that sample esterification before injection is not necessary. The GC method can detect 20 ng/l, which was estimated to be equivalent to approximately two coliforms per 100 ml. Comparison of coprostanol analyses to total and fecal coliform analyses confirmed the predicted advantages of a chemical method over a biological method. Samples were preserved with concentrated sulfuric acid (1 ml/l sample).

The GC method was used in extensive field surveys, degradation studies, and treatment plant efficiency studies. A reasonably good correlation between coprostanol concentration and treatment plant efficiency (BOD, COD, and TOC measurements) was found, but further measurements are needed.

A colorimetric method was developed to determine coprostanol at a concentration of 1 μg/l. Analysis time was estimated to be two hours, but several samples were analyzed simultaneously. Colorimetric analyses of coprostanol in field samples gave higher results than GC analyses; high colorimetric results were probably due to the presence of other fecal steroids. (Alford-EPA)

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ANALYSIS OF COPROSTANOL, AN INDICATOR OF FECAL CONTAMINATION

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1 – page 4

ABSTRACT

The gas chromatographic analysis of coprostanol was improved by showing that it was not necessary to esterify samples prior to injection. A method of sample preservation was developed using one ml of concentrated H_2SO_4 per liter of sample. Field surveys compared coprostanol analyses with total and fecal coliform and confirmed the predicted advantages of a chemical method over a biological method.

The gas chromatographic method was used in extensive field surveys, in degradation studies, in treatment plant efficiency studies and as the standard for evaluation of a colorimetric method. The method was capable of determining twenty nanograms per liter, which was estimated to be equivalent to approximately two coliforms per 100 ml.

An attempt was made to correlate coprostanol concentration with treatment plant efficiency. There was a reasonably good correlation between coprostanol and BOD, COD and TOC. At least enough so that further measurements should be made in a large number of treatment plants.

A colorimetric method has been developed that can be used to determine coprostanol at levels of one μg/l in polluted water. The time required was estimated to be approximately two hours. Several samples could be run simultaneously in the same time. Analysis of field samples gave higher results than GLC but it was shown that the most probable interferants were other fecal steroids.

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CONTENTS

ABSTRACT iii

CONCLUSIONS 1

RECOMMENDATIONS 3

INTRODUCTION 4
Several Potential Chemical Indicators 4

GAS-LIQUID CHROMATOGRAPHY 8
Materials 10
Procedures 11
Chromatography of Free Sterols 12
Resolution of Coprostanol and Cholesterol 13
Extraction and Cleanup 13
Clean-up with Acetonitrile 13
Clean-up with 70 Percent Ethanol and Acetonitrile 16
Clean-up with N,N-dimethylformamide 17
Clean-up with Thin-Layer Chromatography 17
Saponification 18
Wastewater Effluents 18
Activated Sludge Effluent 24
Raw Sewage 25
Trickling Filter Effluent 25
Stability of coprostanol toward Microbial Degradation 27
Procedure 28
Results 28
Activated Sludge 28
Preservation of Samples 36
Field Surveys 38

CORRELATION OF COPROSTANOL CONCENTRATION WITH OTHER MEASURES OF DOMESTIC WASTEWATER TREATMENT PLANT EFFICIENCY 44
Procedure 45
Results 46
Statistical Analysis of Results 52
Discussion of Results 62

DEVELOPMENT OF A COLORIMETRIC METHOD 63
Introduction 63
Recovery of Coprostanol 64
Oxidation of Cholesterol and Coprostanol 67
Formation of Coprostanone-2,4-Dinitrophenylhydrazine and Removal of Unreacted 2,4-DNPH 77
Color Development 79
Summary of Colorimetric Method 96
Applications of Colorimetric Method 97

CONTENTS

Summary of Field Studies 107

ACKNOWLEDGEMENTS 108

REFERENCES 109

APPENDIX 113

FIGURES

1 Planar and Perspective Representations of Cholesterol and Coprostanol 6

2 Structural Differences Between Coprostanol, Cholesterol and Epicoprostanol 7

3 Chromatogram of Free Coprostanol 14

4 Chromatograms Showing Resolution of Cholesterol and Coprostanol 15

5 Environmental Sample--No Clean-up by Thin-Layer Chromatography 19

6 Environmental Sample--Clean-up by Thin Layer Chromatography 20

7 Chromatogram--Extract of Effluent from City of Gainesville Trickling Filter Plant Before Saponification 22

8 Chromatogram--Extract of Effluent from City of Gainesville Trickling Filter Plant After Saponification 23

9 Rate of Change of Coprostanol Concentration in Sample 1 and 2 31

10 Rate of Change of Coprostanol Concentration in Sample 3 and 4 32

11 Total Coliform--Rate of Change in Concentration 33

12 Fecal Coliform--Rate of Change in Concentration 34

13 48-Hour Plate Count--Rate of Change in Concentration 35

14 Chromatogram of Extract of Return Activated Sludge 37

15 Sampling Stations, Sweetwater Creek-Payne's Prairie 40

16 Sampling Stations, University of Florida Sewage Treatment Plant--Lake Alice 41

17 Coprostanol vs COD Campus Plant--Primary Treatment 53

18 Coprostanol vs BOD Campus Plant--Primary Treatment 54

19 Coprostanol vs COD Overall 55

FIGURES

20 Coprostanol vs BOD Overall 56

21 Coprostanol vs TOC Special Study on Municipal Contact Stabilization Plant 57

22 Scheme for the Fractionation 65

23 Oxidation of Sterols and Formation of Coprostanone with the Sarett Reagent at 20°C 70

24 Gas Chromatograms of Oxidation Products with the Sarett Reagent 72

25 Yields of the Oxidation Products of Cholesterol with the Sarett Reagent at 20°C 74

26 Yields of the First Oxidation Product of Cholesterol at Various Temperatures 74

27 Yields of the Second Oxidation Product of Cholesterol at Various Temperatures 76

28 Effect of CrO_3 Dosage on Coprostanone in the Oxidation of Sterols with the Sarett Reagent at 80°C 76

29 Hydrolysis of Coprostanone-2,4-DNPH' with Acetic Acid--Concentrated HCl (1:1) 81

30 Effect of Temperature and Time on the Hydrolysis of Coprostanone-2,4-DNPH' 82

31 Absorption Spectra of the 2,4-dinitrophenylazo--Derivative of 1-(N-naphthyl)-ethylenediamine 84

32 Absorption Spectra of the 2,4-dinitrophenylazo--Derivative of α-Naphthylamine 85

33 Effect of the Periodate Dosage on the Stability of the Azo Compound 87

34 Calibration of 2,4Dinitrophenylhydrazine 88

35 Calibration of Coprostanol (3-5 μg Range) 90

36 Calibration of Coprostanol (0.25-3 μg Range) 91

FIGURES

37 Recovery of Coprostanone by the Combined Procedure of Saponification and Oxidation 94

38 Comparison of the Colorimetric Method Values and GLC Values 99

39 Typical GLC Pattern of the 5 Percent Ethylacetate Fraction of Sewage Samples 100

40 Gas Chromatograms of the 5 Percent Ethylacetate and Ethylacetate Fractions from an Alumina Column 104

41 Gas Chromatograms of Hexane Fraction of Sewage Samples 105

TABLES

1 Survey of Effect of Saponification on the Analysis of Wastewaters 21

2 Effect of Saponification on the Analysis of Effluent from the Activated Sludge Process 24

3 Effect of Saponification on the Analysis of Raw Sewage Influent 25

4 Effect of Saponification on the Analysis of Trickling Filter Effluent 26

5 Data for Sample 1--Trickling Filter Effluent Before Chlorination 29

6 Data for Sample 2--Trickling Filter Effluent Before Chlorination 29

7 Data for Sample 3--University Sewage Plant Effluent After Chlorination 30

8 Data for Sample 4--University Sewage Plant Effluent and Surface Water 30

9 Preservation Study Results 38

10 Survey Data for Sweetwater Creek--Payne's Prairie 42

11 Survey Data for University of Florida Sewage Treatment Plant--Lake Alice 43

12 Data from Campus Plant--Primary Treatment 47

13 Data from Campus Trickling Filter Plant 48

14 Data from Campus Contact Stabilization Plant 49

15 Data from Campus Plant Tertiary Treatment 50

16 Data from Municipal Contact Stabilization Plant 50

17 Data from Municipal Trickling Filter Plant 51

18 Data from the Special Study on the Municipal Contact Stabilization Plant 51

19 Precision Test Results 52

TABLES

20 Summary of Statistical Analyses 58

21 Estimation of Error in Recovery by Solvent Extraction-Washing 66

22 Effect of Saponification 68

23 Effect of Saponificatnon on Preserved Samples 68

24 Elution Pattern on an Alumina Column 75

25 Effect of Extraction Conditions for Coprostanone-2,4-DNPH 78

26 Time and Temperature Effects on Reagent Blanks 78

27 Absorption Intensities and Reagent Blanks of Color Development Techniques in Alkali-Basic Solvent Systems 80

28 Absorption Intensities of the Azo Compounds and the Reagent Backgrounds 86

29 Effect of Pyridine Base Solution on the Recovery of the Azo Compound by Ethylacetate Extraction 92

30 Estimate of Analytical Time 92

31 Color Background of Oxidation Products of Cholesterol 95

32 Separation of Coprostanone and Cholesterol on an Alumina Column 96

33 Coprostanol Concentration of Field Samples 101

34 Statistics of the Overestimation of Coprostanol by the Colorimetric Method 102

CONCLUSIONS

Gas Chromatography

A procedure for analysis of coprostanol was developed that simplified the existing procedures by eliminating both the preparation of the trimethylsilyl ether (TMS) derivative and the saponification step. It was not necessary to prepare the TMS derivative as adequate resolution was accomplished on a six foot glass column packed with Gas Chrome Q using the free sterol. The saponification step could be eliminated as the 10-15 percent increase in quantity did not justify this lengthy step. It was found that acetonitrile could be used to clean-up the hexane extract.

The degradation of coprostanol in sewage plant effluent was more rapid in unchlorinated than in chlorinated samples leading to the conclusion that the degradation was biologically mediated. No correlation was found between coprostanol degradation and either total or fecal coliform counts.

A method of sample preservation was developed using either sulfuric acid or low temperature storage.

Field surveys showed that coprostanol degrades slowly in the environment and does not correlate with either fecal or total coliform levels.

In a study of the correlation of coprostanol concentration with other measures of domestic wastewater treatment plant efficiency a good correlation for a linear relationship was shown between coprostanol and TOC, with a coefficient of determination of 0.977; however, COD and BOD did not correlate very well.

A simple colorimetric method for the analysis of coprostanol was developed. The method eliminated interferences from cholesterol and other constituents by oxidation with CrO_3-pyridine and by simple chromatography on an alumina column. Although the reagent also oxidized coprostanol, the resultant coprostanone formed quantitatively and was separated on the alumina column.

The color was developed as follows: Coprostanone was reacted with 2,4-dinitrophenylhydrazine (2,4-DNPH) to form the hydrazone which was hydrolyzed after the excess hydrazine was removed. The liberated 2,4-DNPH was then oxidized with periodate to the diazonium salt and coupled with α-naphthylamine. The azo compound had intense absorption, with $\varepsilon = 35,000$, at 500 nm in an acidic solution of ethylacetate-acetic acid-concentrated HCl.

The complete analysis required about two hours. The method was applied to several field samples and over-estimated the coprostanol by a factor of 1.6, as compound to results obtained from GLC. The compounds that interfered were shown to have a high probability of being of fecal or urinal origin.

RECOMMENDATIONS

The recommendations can be considered under three basic headings, as they relate to the areas of study.

Gas chromatography--It is recommended that (1) additional studies focus on the preparation of halogenated derivatives that could be detected by electron capture and thus increase the instrumental sensitivity; (2) the ratio of free coprostanol to total coprostanol be determined for a variety of environmental samples in order to evaluate the need for a saponification step, and (3) the correlation of coprostanol with some conservative biological parameter needs to be developed.

Treatment plant efficiency--It is recommended that sufficient studies be conducted to determine the correlation between coprostanol and TOC, COD and BOD for a wide variety of waste treatment plants.

Colorimetry--It is recommended that the method developed in this study be used as widely as possible in field study in order to ascertain its application to a variety of environmental regimes.

INTRODUCTION

Total coliform and fecal coliform have been used as measures of water quality for uses ranging from shellfish waters to public water supplies. Unfortunately, coliform organisms may originate from sources other than feces. Even the membrane filter procedure for fecal coliform gives only 93 percent accuracy for differentiation between coliforms of warm-blooded animals and coliforms from other sources [1]. To obtain good results, one must analyze for coliform within 30 hours after sampling. A minimum of 24 hours are needed for incubation, so there is a delay between sampling and knowledge of fecal pollution. Chlorinated sewage effluents often show low coliform counts even though water quality is poor. Because of the weaknesses of the coliform tests, research has continued in the search for indicators of fecal pollution.

Several Potential Chemical Indicators

1. Uric Acid--Uric Acid has been used as a Pollution indicator [2,3]. The amount of uric acid in a specimen is measured by observing the reduction in absorbance at 292 nm wavelength resulting from its oxidation by the enzyme uricase. Uric acid is the principal vehicle of nitrogen excretion for reptiles, birds and insects, and is the end-point of purine metabolism in man, the higher apes and Dalmatian dogs [4]. However, 95 percent or more of the uric acid is degraded by conventional treatment processes and no uric acid could be detected in the Ohio River even in samples taken near the outfall of the Little Miami River Sewage Plant [2]. This indicates that only recent pollution of a stream with raw waste water could be detected by use of uric acid.

2. Cholesterol--Cholesterol has been suggested as an indicator of fecal pollution, and the concentration of cholesterol in natural waters has been measured [5]. Since it is known that cholesterol in surface waters could come not only from excreta, but also from eggs, milk, lard, wool, grease, etc., cholesterol can not be considered a specific indicator of fecal pollution. In seawater, cholesterol is evidently present naturally at concentrations close to its water solubility [6,7]. The origin of this cholesterol is unknown. Nonetheless, the use of cholesterol as an indicator in fresh water may be useful; although it would give false positive results, a more serious problem in assessing water safety.

3. Coprostanol--Coprostanol, or 5β-cholestan-3β-ol, has shown promise as an indicator of fecal pollution [5,8,9]. This sterol is a characteristic fecal organic compound which is found in the feces of higher animals, including man [10]. Recently it has been claimed that coprostanol is present in whale oil [11]. For all practical purposes, the only apparent source of coprostanol is the feces of the higher animals. Under normal conditions, coprostanol is the major fecal neutral steroid. In addition, the fecal neutral steroid fraction contains vary-

ing but usually small amounts of the following steroids of endogenous orgin: cholesta-5,7-dien-3β-ol (7-dehydrocholesterol), cholesterol, 5α-cholest-7-en-3β-ol (lathosterol), 5β-cholest-7-en-3β-ol, 4α-methyl-5α-cholest-7-en-3β-ol (methosterol), 5α-cholestan-3β-ol (cholestanol), 5β-cholestan-3α-ol, 5α-cholestan-3-one, and 5β-cholestan-3-one [12]. It has been claimed that a small part of the sterols is present in the form of esters [13,14,15].

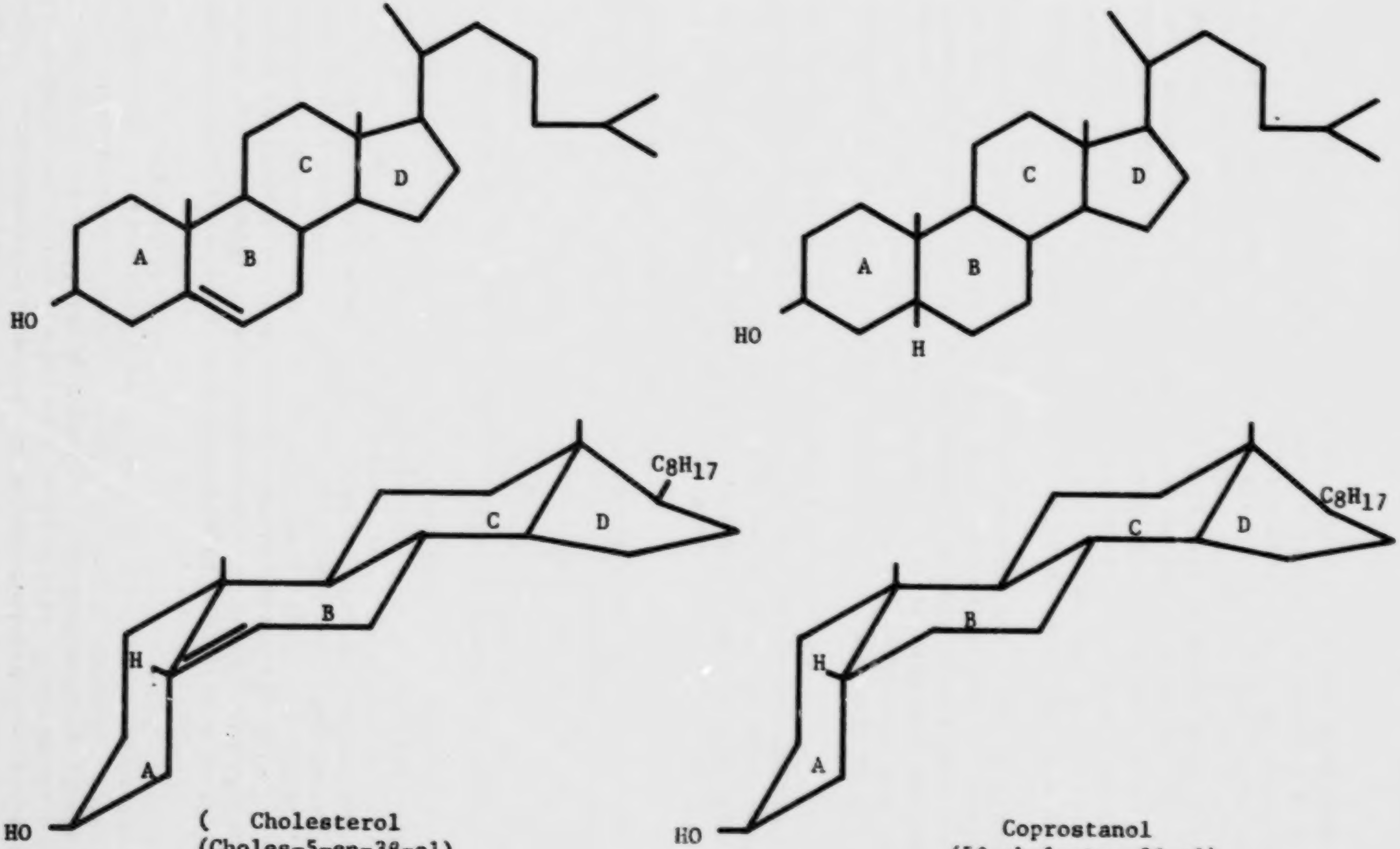
The microbial conversion of cholesterol to coprostanol in the large intestines of mammals has been demonstrated [16,17] and the mechanism of the conversion process has been investigated [18,19,20]. The major reaction mechanism has been found to be a direct saturation of the double bond mediated by intestinal microoganisms. The pathway for formation of coprostanol esters has been investigated and the conclusion was reached that the main pathway was an esterification of free 5β-cholestan-3β-ol and not a hydrogenation of cholesteryl esters [21]. Figure 1 shows the structures of cholesterol and coprostanol.

Studies have shown that the fecal excretion of neutral fecal steroids in human subjects fed standardized diets containing either saturated or unsaturated fat ranges from 500-700 mg per day [22-27], the majority of which consists of coprostanol, cholesterol and their esters. However, studies of the concentration of coprostanol in sewage indicate concentrations of coprostanol corresponding to about two grams of coprostanol per capita per day [5].

Coprostanol was first isolated from human feces by Austin Flint, Jr., in 1862. The pharmacologist von Bondzyński characterized the sterol as an alcohol of the formula $C_{27}H_{48}O$ and gave it the name coprosterol (from the Greek "Kopros," dung). The name was later changed to coprostanol for uniformity of nomenclature.

Coprostanol is the C_5-epimer of cholestanol. The typical saturated sterols found in nature are A/B trans or 5 α compounds, and the only exception to this rule is coprostanol. It is said that epicoprostanol (5β-cholestan-3α-ol) is thermodynamically more stable than coprostanol because of steric strain from α axial hydrogens [28]. Structures of cholestanol, coprostanol and epicoprostanol are shown in Figure 2.

Coprostanol is a white crystalline solid at room temperature, showing a melting point of 101°C. A variety of acids form esters with coprostanol, including the acetate, propionate and benzoate which exhibits melting points of 89-90°, 99-100° and 124-5°C respectively. It is very soluble in ethanol, ether, benzene,and chloroform, and slightly soluble in methanol [29]. It is only very slightly soluble in water. No values for its solubility in water are given in the literature, but values for a structurally similar sterol, cholesterol, have been given as 2.6×10^{-8} g/ml at 30.0°C, which corresponds to 26 μg/l [30]. Coprostanol would be expected to show a similar solubility in distilled water. However, in primary sewage effluent, values have been reported



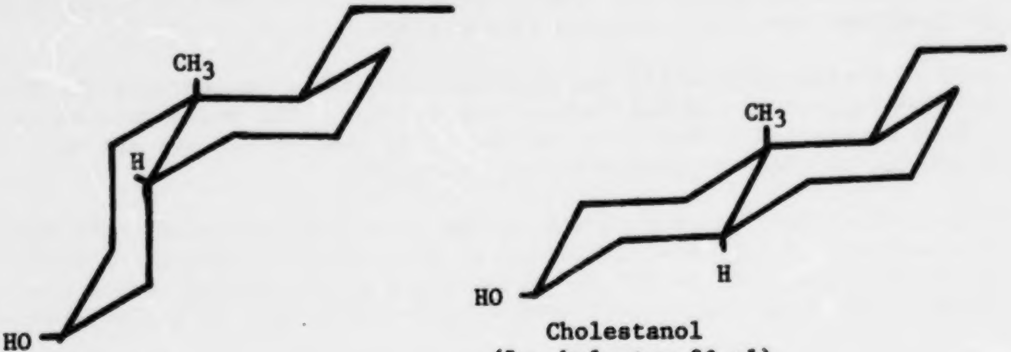
(Cholesterol
(Choles-5-en-3β-ol)

Coprostanol
(5β-cholestan-3β-ol)

FIGURE 1. PLANAR AND PERSPECTIVE REPRESENTATIONS OF CHOLESTEROL AND COPROSTANOL

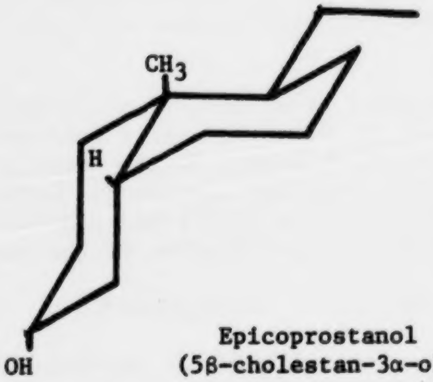
as high as 260 μg/l for cholesterol and 750 μg/l for coprostanol [5], which indicate that the amounts of cholesterol and coprostanol found in sewage are not limited by their solubilities and that much of the cholesterol and coprostanol is present combined with particulate matter.

Coprostanol has been analyzed by colorimetric methods as well as by gas-liquid chromatography [31-34], but the colorimetric methods previously used suffered from a lack of sensitivity as well as the ever-present possibility of spectral interferences. Gerson [33] considered a colorimetric method, which employed a color reagent consisting of ferric chloride--acetic acid--concentrated sulphuric acid. It was not suitable for the determination of less than 75 μg of coprostanol. This amount would suffice for the analysis of coprostanol in 1-2 liters of primary treated sewage, but, in many cases, secondary treated sew-



Coprostanol
(5β-cholestan-3β-ol)

Cholestanol
(5α-cholestan-3β-ol)



Epicoprostanol
(5β-cholestan-3α-ol)

FIGURE 2. STRUCTURAL DIFFERENCES BETWEEN COPROSTANOL, CHOLESTANOL, AND EPICOPROSTANOL

age contains coprostanol in concentrations too low to be detected by Gerson's method. The absorption peak, measured at 440 nm, may exhibit interferences, since sewage is a complex mixture containing many compounds that may absorb at 440 nm. Thus, a clean-up step involving digitonide precipitation and/or thin-layer chromatography (TLC) would be necessary.

Despite the difficulties involved in developing a colorimetric method for detecting coprostanol in the environment, it would be well worth the effort. A colorimetric method for cholesterol that is capable of detecting amounts as small as 2 μg has been developed [31]. A similar method for coprostanol may be feasible. Advantages of a colorimetric method would be more rapid analysis, less expensive equipment and less technical training. It may be possible to develop a colorimetric test for total sterols, which could be used to measure the efficiency of sewage treatment processes, since coprostanol is removed in a properly designed and operated activated sludge plant.

Another possibility is the use of liquid-liquid chromatography (LLC) for the separation and analysis of coprostanol. LLC would seem to be a good separation method, but the detectors used in LLC are not as sensitive as those available for use with GLC.

This project has centered on GLC as the principal analytical tool for the analysis of coprostanol because of the excellent separation that is possible, as well as for the sensitivity of the method, which can detect concentrations of coprostanol less than 1 μg/l in the environment.

Samples have been taken in both glass [28] and plastic [9] containers. Glass containers would seem to be preferable since adsorption onto glass is less likely than adsorption onto plastic. There have been no reported studies on the preservation of water samples for the determination of coprostanol. Indeed, no mention has been made that preservation may be necessary.

The purpose of the present study was: (1) to simplify the gas-liquid chromatographic method for the analysis of coprostanol and thus reduce the time necessary for an analysis, (2) to develop a colorimetric method of analysis, (3) to evaluate the use of coprostanol concentration as a measure of treatment plant efficiency and (4) to evaluate coprostanol as measure of fecal contamination in the environment.

GAS-LIQUID CHROMATOGRAPHY

The previous studies on the analysis of coprostanol in the environment have used slightly different techniques for extraction and clean-up, prior to gas-liquid chromatography (GLC)[5,8,9].

Murtaugh and Bunch were the first to analyze for coprostanol in the environment and used a procedure which consisted of extraction with hexane and clean-up by thin-layer chromatography (TLC) [5]. Smith and Gouron also extracted water samples with hexane and used a clean-up by TLC [8].

Black, Singley and Nordstrand [9] found it only necessary to extract water samples with chloroform, evaporate the solvent, and redissolve the residue in chloroform prior to analysis by gas-liquid chromatography. No clean-up by TLC was found necessary for the samples they analyzed.

Rosenfeld's experimental technique involved a preliminary separation by column chromatography on alumina, followed by a saponification of the esters and quantification by GLC.

On the basis of the evidence for the existence of coprostanol esters, Murtaugh and Bunch included a saponification step in the analytical procedure for coprostanol. Samples were refluxed for three hours in a solution of 7.5 percent potassium hydroxide in 70 percent ethanol after initial extraction with hexane and prior to clean-up by TLC.

Other investigators have omitted the saponification step [8,9]. No reasons were given for the omission. Evidently, either the step was considered too time-consuming or the fraction present as esters was considered too small to need recovery.

The basic technique for gas-liquid chromatographic sepatation of the steroids was established by Vandeheuvel et al. in 1960 [35]. The technique used a column of 2-3 percent SE 30 on 80-100 mesh Chromosorb W. A number of steroid compounds including hydrocarbons, ketones, alcohols, ether and acetyl esters were found to separate at 222°C with no sign of decomposition.

The technique of Vandenheuvel was applied to the analysis of fecal neutral steroids [36]. Some investigators found that the use of trimethylsilyl ether derivatives of the sterols gave sharper peaks and increased resolution [36,37]. The method of Vandenheuvel has been used, with certain modifications, by all of the investigators of coprostanol in environmental water samples.

Murtaugh and Bunch used a one-eighth inch by five foot stainless steel column packed with 60-80 mesh Chromosorb W coated with 5 percent SE 30. Oven temperature was 235°C and injector port and detector were both kept at 260°C. Helium carrier gas was used at a flow rate of 40 ml/minutes. Trimethylsilyl ether derivatives of all samples were prepared prior to injection onto the column. Quantitative determinations were based on peak height compared to a standard curve prepared from known reference standards.

Smith and Gouron used a 6mm by 1.83 m glass tube packed with 3 percent

SE 30 on 80-100 mesh Gas-Chrom Q. Analyses were conducted using Hewlett-Packard Corp. F and M Models 400 and 402 gas chromatographs equipped with hydrogen flame detector systems. Column temperature was 230°C and injection port was maintained 260°C. Nitrogen gas was used as carrier at a flow rate of 20 ml/minute. Free sterols were chromatographed, but no quantitative determinations were made.

Black et al. used a 4 mm by 4 foot glass U tube packed with 3 percent OV17 on 60-80 mesh Gas-Chrom Q. A Research Specialties Company Model 600 gas chromatograph equipped with a Sr ionization detector was used for all analyses. Argon was used as carrier gas with inlet pressure being maintained at 30 lb/inch. Column temperature was maintained at 240°C or programmed from 150-240°C, and the injection port was preheated to 270°C. Peak heights obtained from the chromatography of standard solutions were used for quantitative analysis. Free sterols were chromatographed and found satisfactory. However, for verification of coprostanol, silyl ether derivatives were prepared on column by bis-(trimethylsilyl)acetamide.

Materials

All solvents used in his study were reagent grade or its equivalent. Pesticide quality hexane (Matheson, Coleman and Bell) was used as the extracting solvent. Reagent grade 95 percent ethanol as well as acetonitrile (Mallinckrodt Chemical) were used in the clean-up procedure.

Coprostanol was supplied in pure form by K and K Laboratories, Inc., Plainview, New York, as well as by Applied Sciences, Inc., State College, Pennsylvania. Cholesterol was obtained from K and K Laboratories, Inc., and cholestane was purchased from Applied Sciences, Inc. Coprostanol was judged pure on the basis of melting point determination as well as gas chromatographic data.

Membrane filter media (Bacto-m Endo Broth MF, Bacto-m FC Broth Base and Bacto-Rosolic Acid) were purchased from Difco Laboratories, Detroit, Michigan. Disposable plastic petri dishes (48 x 8.5 mm) were obtained from Millipore Corporation, Bedford, Massachusetts (see Figure 3).

Three percent SE-30 on Gas-Chrom Q served as liquid and support phase for gas chromatography. Three percent QF1 on Gas Chrom-Q was used for confirmatory analyses. Gas-Chrom Q is an acid washed, silanzied, diatomaceous earth.

Glass sampling bottles were used for all samples. Extraction was done in two-liter separatory funnels equipped with Teflon stopcorks to avoid contamination from stopcork grease. Either a Büchi Evaporator (Rinco Instruments Company) or a Buchler Flash Evaporator (Buchler Instruments) were used to reduce the volume of hexane extracts. A 500 ml Kuderna-Danish Evaporative Concentrator equipped with a 5 ml receiver was used to collect the reduced volume of hexane (Ace Glass Company). With this apparatus it was possible to remove the 5 ml receiver with the concentrated hexane extract and thus avoid loss of extract on transfer. A 10 μl syringe (#701 syringe Hamilton Company, whittier, California) was used for gas chromatography. Six feet by one-fourth inch coiled glass columns (Applied Sciences) were employged in gas chromatography.

A. F. & M. Corporation Model 810 gas chromatograph, equipped with both flame ionization and Ni electron capture detectors was used. The recorder was a Sargen Model SR, reading one millivolt full scale.

Procedures

Most extractions of water samples were done in the following manner. Exceptions are noted in discussion of specific procedures. One liter water samples were taken, and 2 ml HCl (conc.) and 5 ml 20 percent NaCL were added. The solution was extracted with two 50 ml portions of hexane. The hexane extracts were combined and washed with two 25 ml portions of 70 percent ethanol followed by two 25 ml of acetonitrile saturated with hexane. The hexane was reduced to near dryness on a flash evaporator. The Kuderna Danish Evaporative Concentrator was then washed with a small amount of hexane and the concentrated extract was colleted in the 5 ml receiver. The receiver was removed and the hexane was evaporated to dryness with a stream of dry air. After redissolving in a known amount of hexane the sample was ready for gas chromatography.

To correct injection errors, the following technique of filling the microsyringe was used. First a small amount of air was drawn into the syringe. The syringe was held down and then the sample liquid was drawn in followed by more air. This gave a sandwich of liquid sample between two slices of air. The volume of the liquid sample could be read accurately by means of the gradations of the barrel. After the needle was thrust through the septum, the plunger could be pushed all the way into the barrel. The air ahead of the sample permitted accurate measurement of the volume of contained liquid and the air behind the liquid permitted all of the sample to be injected into the chromatograph.

General conditions for gas chromatographic analyses were as follows: Any variations on these general conditions are specified under experimental procedures.

Column temperature was kept at 250°C, and injector port and flame ionization detector were maintained at 270°C. Nitrogen gas flow rate was approximately 40 ml/minute. A 6 foot by 1/4 inch coiled glass column of three percent SE 30 on Gas-Chrom Q was for all analyses except those noted otherwise.

Coliform analyses were done using the Membrane Filter technique. Standard procedures, as outline in the 13th edition of Standard Methods for the Examination of Water and Wastewater [1], were used on all analyses.

The procedure used for analysis of coprostanol to date was somewhat long and complicated. Therefore, a series of studies were initiated on modification or elimination of some of the steps.

Either hexane or chloroform may be used to extract coprostanol from water samples [5,8,9]. Chloroform will extract more non-lipid materials from sewage than will hexane [38,39,40]. Hexane is a more selective solvent than chloroform, since it will extract mostly non-polar material while chloroform also extracts many polar substances.

Acetonitrile, as well as dimethylformamide, was used in pesticide analyses to remove the chlorinated hydrocarbons from lipid-like material [41,42]. The lipid-like material remains in the hexane layer, while most pesticides, including the chlorinated hydrocarbons, are extracted into the acetonitrile or dimethylformamide. It may be possible to adapt the procedure for coprostanol by analyzing for the lipids in the hexane layer, and discarding the acetonitrile or dimethylformamide containing undesired substances.

There is disagreement over the necessity of preparing the trimethylsilyl (TMS) ether derivative of coprostanol prior to gas chromatography. Murtaugh and Bunch [5] prepared the TMS derivative, while others [8,9] found that the free sterol gave good results.

The limit of detection of coprostanol using a flame ionization detector was approximately 20 nanograms [5]. In environmental samples, this limit of detection is difficult to achieve because of interferences from other substances present in the water.

Picogram amounts of steroids have been analyzed by formation of the heptafluorobutyrate derivatives and detection using an electron capture (EC) detector [43,44,45].

Chromatography of Free Sterols

Summary of Results

No extraneous peaks were found on any of the chromatograms of free coprostanol, and since the peaks obtained were sharp with only a small amount of tailing, it was not necessary to prepare the trimethylsilyl ether (TMS) derivatives of coprostanol prior to gas chromatography.

Two conditions must be met in order to chromatograph free sterols. First, an all-glass column should be used, since metal columns have been known to cause breakdown of steroids. Copper, in particular, will catalyze the decomposition. Deactivation is achieved by silanizing the solid support. Gas Chrom-Q is an acid-washed, silanized support of diatomaceous earth.

In order to use peak height as a quantitative measure of coprostanol, there should be sufficient resolution of coprostanol from cholesterol, since cholesterol is invariably found with coprostanol in the environment. Complete resolution was not necessary. Figure 4 showed that the required resolution of cholesterol was acheived by use of a 6 foot column. If one wishes to increase resolution of cholesterol and coprostanol, it can be done by increasing the length of the column, lowering the column temperature, lowering the percent liquid phase on the column, adjusting the flow rate of the carrier gas, or changing to a more selective liquid phase.

The formation of the heptafluorobutyrate derivative of coprostanol evidently does not take place as easily as reported in the literature for plasma estrogen and plasma testosterone. Klyne stated that for reactions dependent on accessibility of x (esterification and hydrolysis of esters) equatorial reacts more rapidly than axial. Since the configuration of the OH group in coprostanol is axial, the reaction may need more drastic conditions than those reported in the literature for the estrogens and testosterone.

Several injections of free coprostanol, dissolved in hexane, were made. Column conditions were as described previously. A typical chromatogram of coprostanol is shown in Figure 3.

Resolution of Coprostanol and Cholesterol

Chromatographs were obtained for coprostanol, cholesterol and combined coprostanol and cholesterol. Cholestane was used as an internal standard to compare locations of peaks. Figure 4 shows that the coprostanol and cholesterol peaks overlap somewhat but the peak height for coprostanol was the true peak height when cholesterol was also present.

Extraction and Clean-up

Summary of Results

N,N-dimethylformamide cannot be used to clean-up the hexane extract, since some of the coprostanol was extracted into the dimethylformamide. Acetonitrile was much more efficient in cleaning up the coprostanol extract. Indications were that very little of the coprostanol was removed by the acetonitrile. Since acetonitrile has a potential for extracting some of the interferents in the GLC analysis for coprostanol, a washing with acetonitrile was used in all subsequent analyses.

Clean-up with Acetonitrile

Fifty μg of coprostanol was dissolved in one liter of distilled water, and the water was then extracted with two 50 ml portions of hexane

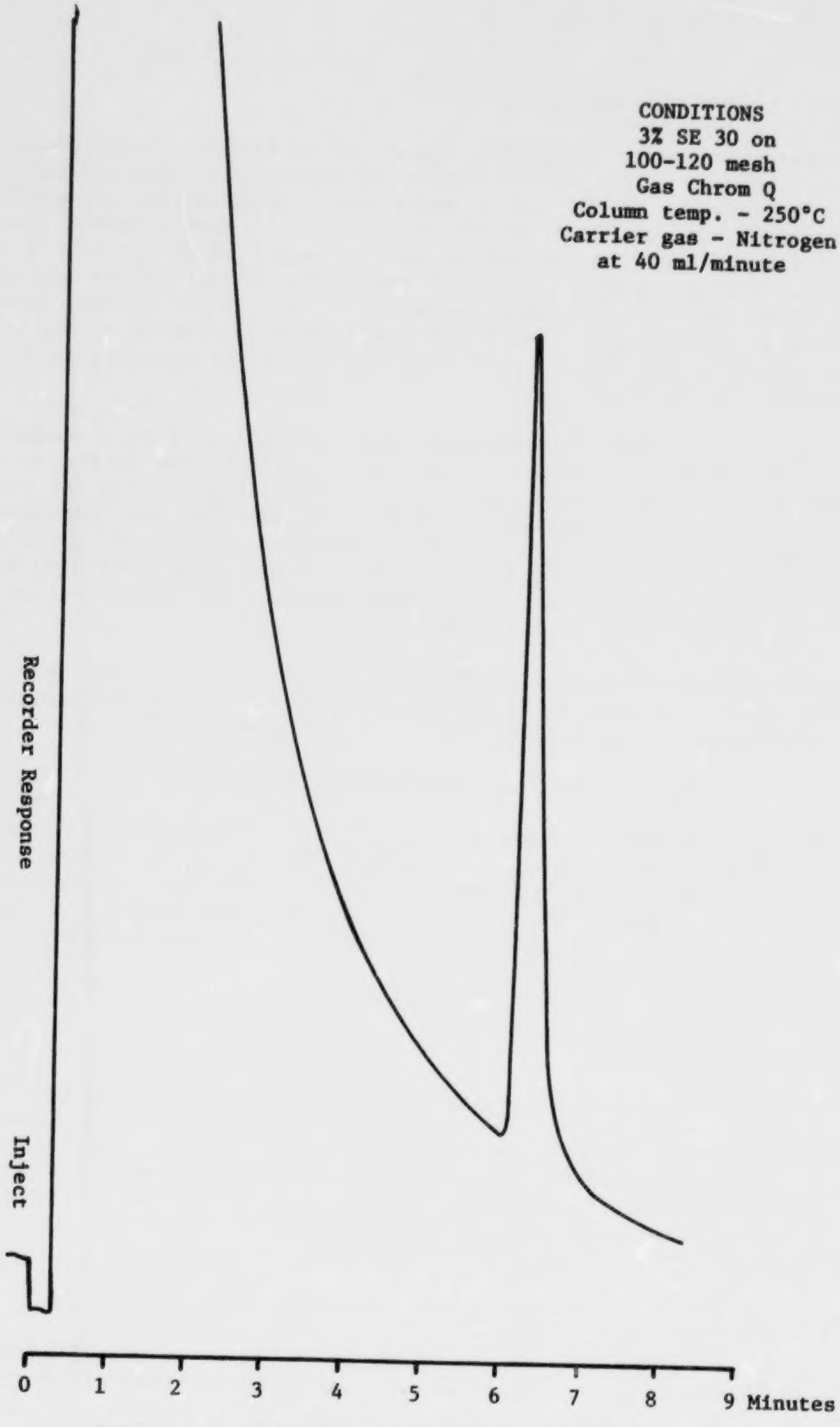


FIGURE 3. CHROMATOGRAM OF FREE COPROSTANOL

CONDITIONS
3% SE 30 on 100-120 mesh Gas Chrom Q
6 foot glass column
Column temp. - 250°C
Carrier gas - Nitrogen at 40 ml/minute

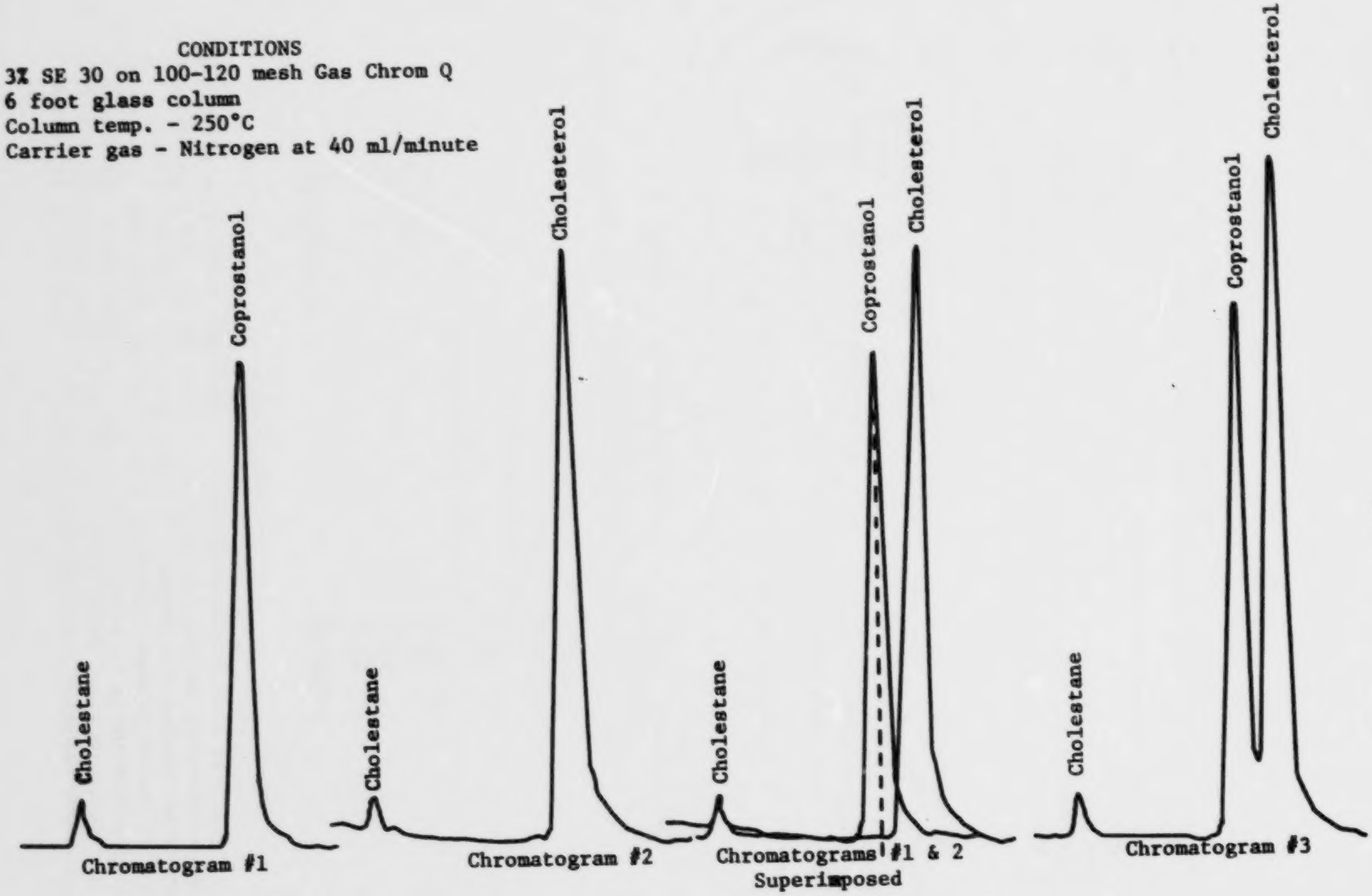


FIGURE 4. CHROMATOGRAMS SHOWING RESOLUTION OF CHOLESTEROL AND COPROSTANOL

(shaken for 2 minutes). The hexane extracts were combined and washed with two 25 ml portions of acetonitrile (shaking for 2 minutes). The acetonitrile washings were combined and reduced by flash evaporation to one ml. The hexane extracts were similarly reduced to one ml. Hexane extract, acetonitrile washings and standard coprostanol were run on a column of 3 percent QF 1 on Gas Chrom Q. Column temperature was 250°C and both inlet and flame ionization detector were at 270°C.

Hexane Extract

µl Injected		Peak Height
3.5		4.78 cm
3.5		4.55
3.5		4.20
		4.51 Avg.
	Acetonitrile Washings	
3.5		0.14
	Reference Coprostanol	
3.5		4.66
3.5		4.54
		4.60 Avg.

% Recovery of coprostanol in hexane $= \frac{4.51}{4.60} \times 100 = 98.1\%$

% Recovery of coprostanol in acetonitrile $= \frac{0.14}{4.60} \times 100 = 3.04\%$

Total Recovery $= 98.1 + 3.04 = 101.1\%$

Clean-up with 70 Percent Ethanol and Acetonitrile

Forty µg coprostanol was added to one liter of distilled water. Two ml HCl (conc.) and 5 ml 20 percent NaCl were added to the solution. The water sample was extracted with two 50 ml portions of hexane (shaking for 2 minutes) and then washed with two 25 ml portions of 70 percent ethanol, followed by two 25 ml portions of acetonitrile. The combined hexane extracts were evaporated to dryness and then redissolved in 0.5 ml benzene.

The above procedure was repeated on two more samples and GLC was run on the three extracts, using a column of 3 percent SE 30 on Gas Chrom Q. Column temperature was 250°C, inlet temperature 270°C, and flame ionization detector temperature was 270°C. A standard sample of coprostanol was prepared by dissolving 40 µg coprostanol in 0.5 ml benzene. Three-aliquot samples of the standard solution were run on GLC.

Data are given in terms of peak height obtained per 1 µl injected into the column from a total sample of 0.5 ml.

Peak Height/μl Injected Standard Solution	Peak Height/μl Injected Samples
3.93 cm/μl	3.63 cm/μl
4.06 cm/μl	3.82 cm/μl
3.74 cm/μl	3.63 cm/μl
3.91 cm/μl Avg.	3.69 cm/μl Avg.

$$\% \text{ Recovered} = \frac{3.69}{3.91} \times 100 = 94.4\%$$

The 95 percent C.I. can be calculated as $\mu = \bar{x} \pm t_{n}-1\ (\alpha/2)\ 5/\gamma m$

$$= 94.4 \pm \frac{4.3\ (2.05)}{3}$$

$$= 94.4 \pm 5$$

Clean-up with N,N-dimethylformamide

Five hundred μg of coprostanol was added to 50 ml hexane and the hexane was washed with two 25 ml portions of N,N-dimethylformamide. The hexane was washed with one 25 ml portion of distilled water to remove all traces of N,N-dimethylformamide. The hexane was evaporated to dryness by flash evaporation and the dried residue was redissolved in one ml. The procedure was repeated on another sample. A column of 3 percent SE 30 on Gas Chrom Q was used. Column temperature was 250°C and inlet and detector were 270°C.

Peak Height/μl Injected Standard	Peak Height/μl Injected Sample 1	Peak Height/μl Injected Sample 2
2.19 cm/μl	0.754 cm/μl	0.753 cm/μl
2.55 cm/μl	0.705 cm/μl	0.748 cm/μl
2.24 cm/μl	0.808 cm/μl	0.810 cm/μl
2.09 cm/μl	0.756 cm/μl Avg.	0.770 cm/μl
2.31 cm/μl		
2.28 cm/μl Avg.		

% Recovery

$$\text{Sample 1} = \frac{0.756}{2.28} \times 100 = 33.2\%$$

$$\text{Sample 2} = \frac{0.770}{2.28} \times 100 = 33.8\%$$

Clean-up with Thin-Layer Chromatography (TLC)

A number of environmental water samples were run to determine the necessity of TLC as a preliminary clean-up step. It was found that TLC was necessary for samples with concentrations below approximately 2 μg/1, using one-liter samples, but TLC may be omitted on samples with higher coprostanol concentrations. Figure 5 shows a chromatogram of an environmental sample with no prior clean-up by TLC while Figure 6 shows a chromatogram prior to clean-up of a sample which required clean-up by TLC.

Saponification

Saponification is a time-consuming step in the analytical scheme, requiring three hours out of a total analysis time of four hours. The saponification step could be omitted if the amount of coprostanol in the form of esters was small.

Wastewater Effluents

Summary of Results

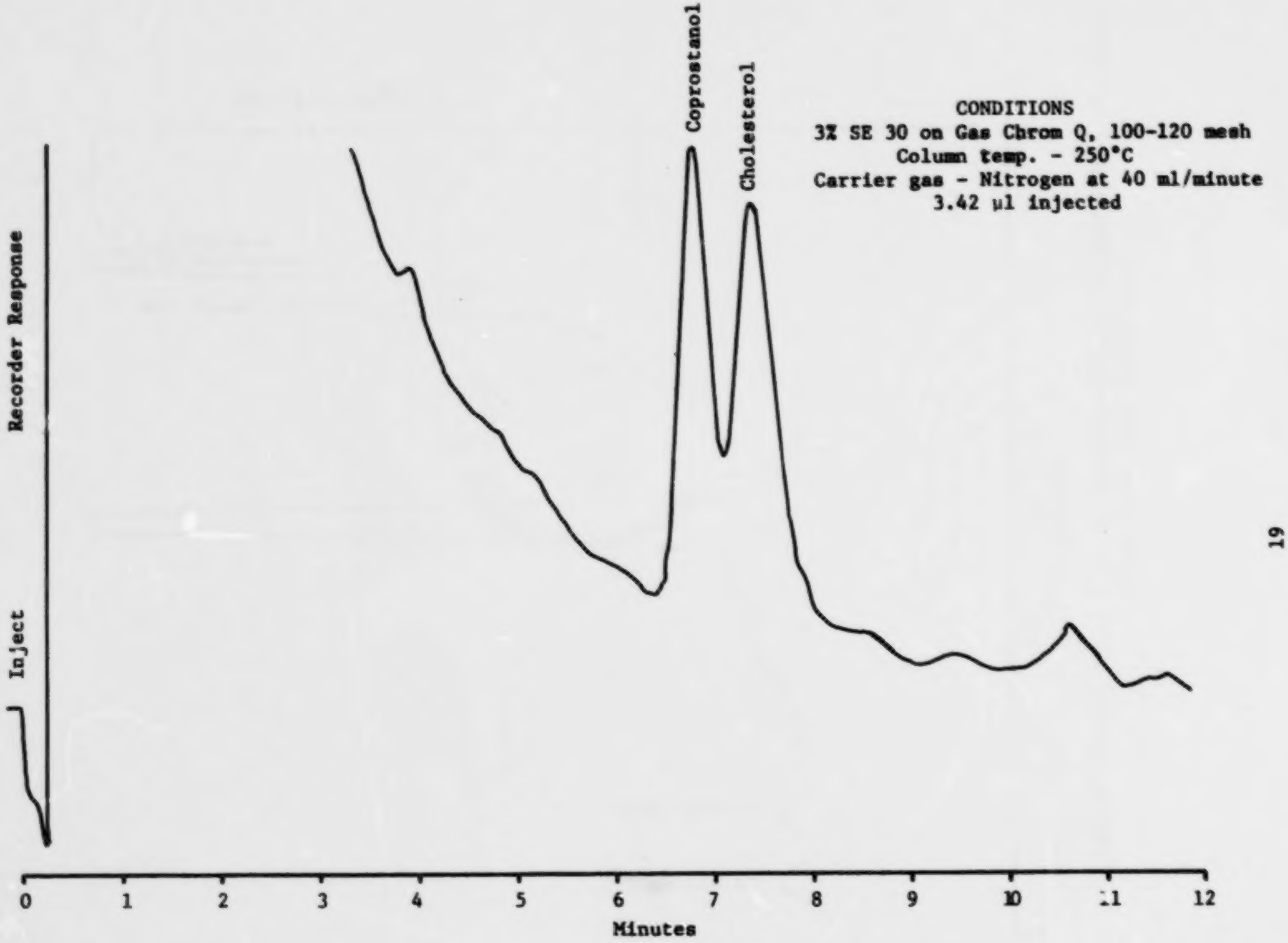
The initial survey of the effect of saponification on the analysis of wastewaters showed no statistical difference between the saponified and unsaponified samples. Since only one sample was taken from each source, three more experiments were performed, refining the experimental design. In these three experiments, 10 repetitions from each source were analyzed before and after saponification. It was found that saponification had a slight effect on the quantitative determination of coprostanol. However, the difference was slight, on the order of 10-15 percent. Such a slight difference does not seem to justify a three-hour saponification step in a procedure that takes four hours to complete the total analysis. Without saponification, analysis time was only one hour. Since the purpose of the analysis is to detect fecal pollution, it would seem that free coprostanol could do this as well as total coprostanol, including the esters of coprostanol. The ratio of free coprostanol to total coprostanol needs further investigation for a variety of samples.

The esterification of sterols in feces requires an enzyme system, probably of bacterial origin. It is highly unlikely that extensive esterification of coprostanol could occur in the natural environment.

Procedure

Samples were obtained from three wastewater treatment plants in the Gainesville, Florida area. The three plants sampled were the City Plant (2.7 million gallons per day [MGD], contact stabilization and 2.2 MGD, trickling filter), the University Plant (1.3-1.8 MGD, contact stabilization and 0.7 MGD, trickling filter), and the Sunland Training Center Plant (0.3 MGD, trickling filter).

Sample volumes varied from 125 ml to 1000 ml. Two samples were taken simultaneously from each location. A 500 ml and a 1000 ml sample were extracted with two 50 ml portions of hexane, and the combined hexane extracts were then washed with two 25 ml portions of 70 percent ethanol and one 25 ml portion of acetonitrile.



CONDITIONS
3% SE 30 on Gas Chrom Q, 100-120 mesh
Column temp. - 250°C
Carrier gas - Nitrogen at 40 ml/minute
3.42 µl injected

FIGURE 5. ENVIRONMENTAL SAMPLE--NO CLEANUP BY THIN LAYER CHROMATOGRAPHY
(Calculated Conc. 24.3 µg/l)

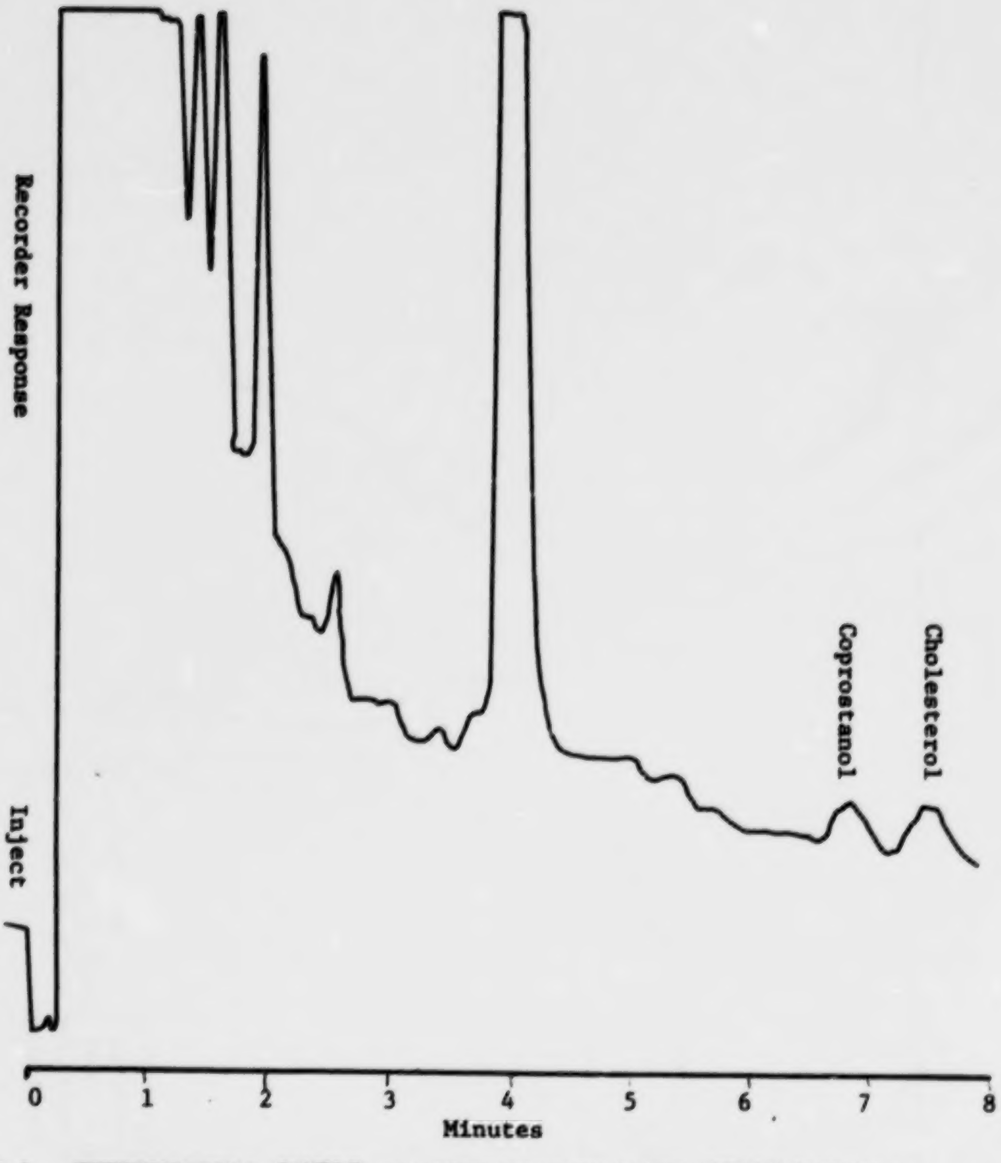


FIGURE 6. ENVIRONMENTAL SAMPLE--CLEANUP BY THIN LAYER CHROMATOGRAPHY
(Calculated Conc. 0.22 μg/1)

The hexane extracts were evaporated on a rotary evaporator using a 500 ml Kuderna-Danish evaporative concentration with a 5 ml receiver. Results of these analyses are shown in Table 1. Figures 7 and 8 show typical chromatograms from samples before and after saponification.

TABLE 1

Survey of Effect of Saponification on the Analysis of Wastewaters

	μg/l Coprostanol No Saponification	μg/l Coprostanol Saponified
City Influent	448	493
University Influent	340	412
Sunland Influent	755	741
City Effluent (Trickling Filter)	91.5	84.6
University Effluent (Trickling Filter)	72.6	70.0
Sunland Effluent (Trickling Filter)	181	168
City Effluent (Contact Stabilization)	16.1	9.1
University Effluent (Contact Stabilization)	1.8	1.0

Statistical Analysis

$H_o \equiv \mu\ \%_D = 0$

$H_A \equiv \mu\ \%_D \neq 0$

$\alpha = 0.2$

Sample	No Sapon.	Sapon.	% D	(% D)^2
1	448	493	-10.04	100.8
2	340	412	-21.17	448.2
3	755	741	+ 1.85	3.42
4	91.5	84.6	+ 7.54	56.9
5	72.6	70.0	+ 3.58	12.8
6	181	168	+ 7.18	51.5
7	16.1	9.1	+43.47	1,890
8	1.8	1.0	+44.44	1,975
			76.85	4,538.62

$\overline{\%\ D} = 9.6$

Therefore $\sigma_{\%D} = \sqrt{\frac{1}{7}\left[\Sigma(\%D)^2 - \frac{(\Sigma\%D)^2}{8}\right]} = \sqrt{542.9} = 23.3$

Then $t = \frac{\overline{\%D} - 0}{\sigma\%D/\sqrt{n}} = 1.16$

From standard statistical tables, two-sided $t_{20\%} = 1.415$

Not significant therefore H_o accepted

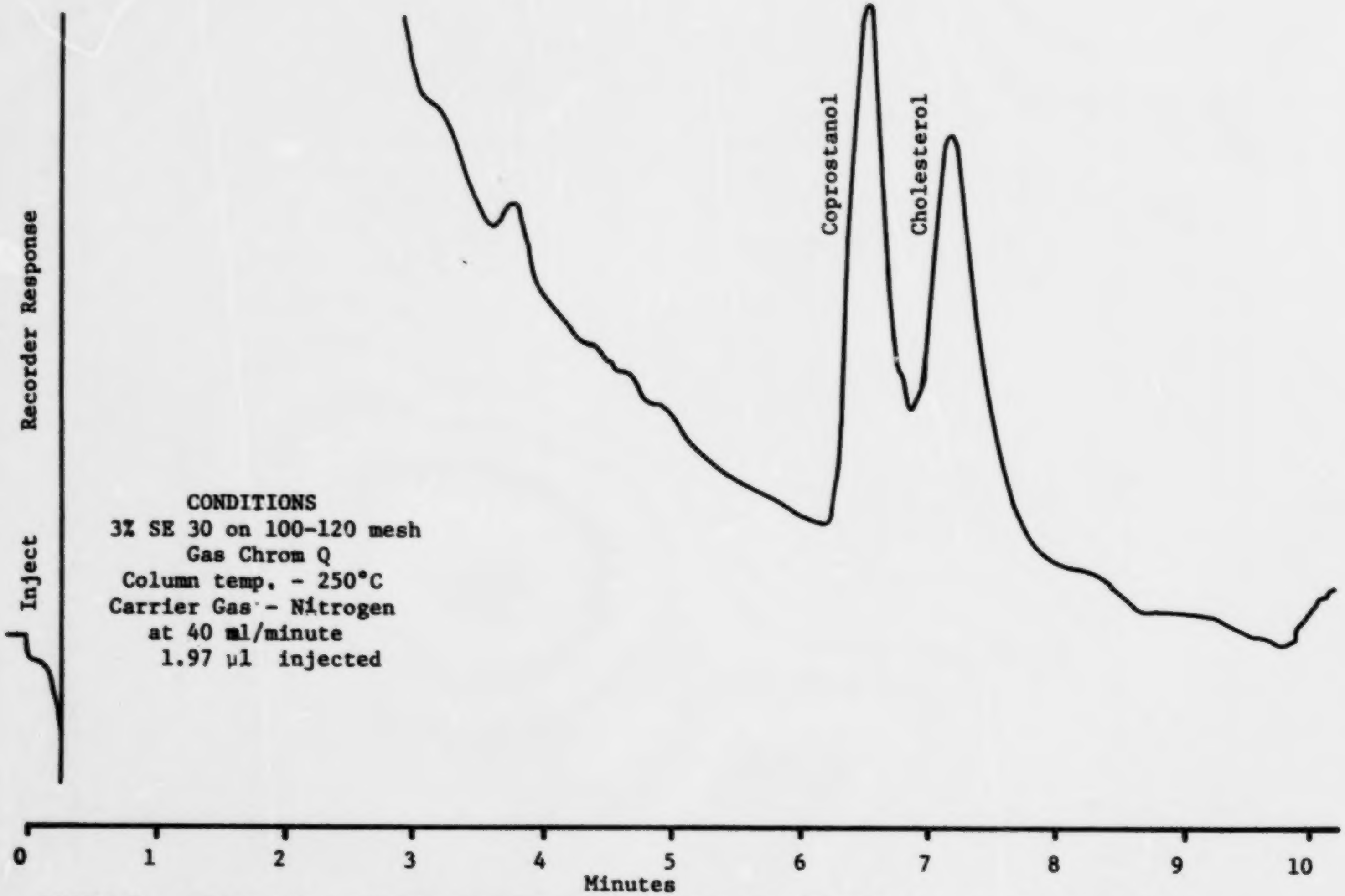


FIGURE 7. CHROMATOGRAM EXTRACT OF EFFLUENT FROM CITY OF GAINESVILLE TRICKLING FILTER PLANT BEFORE SAPONIFICATION

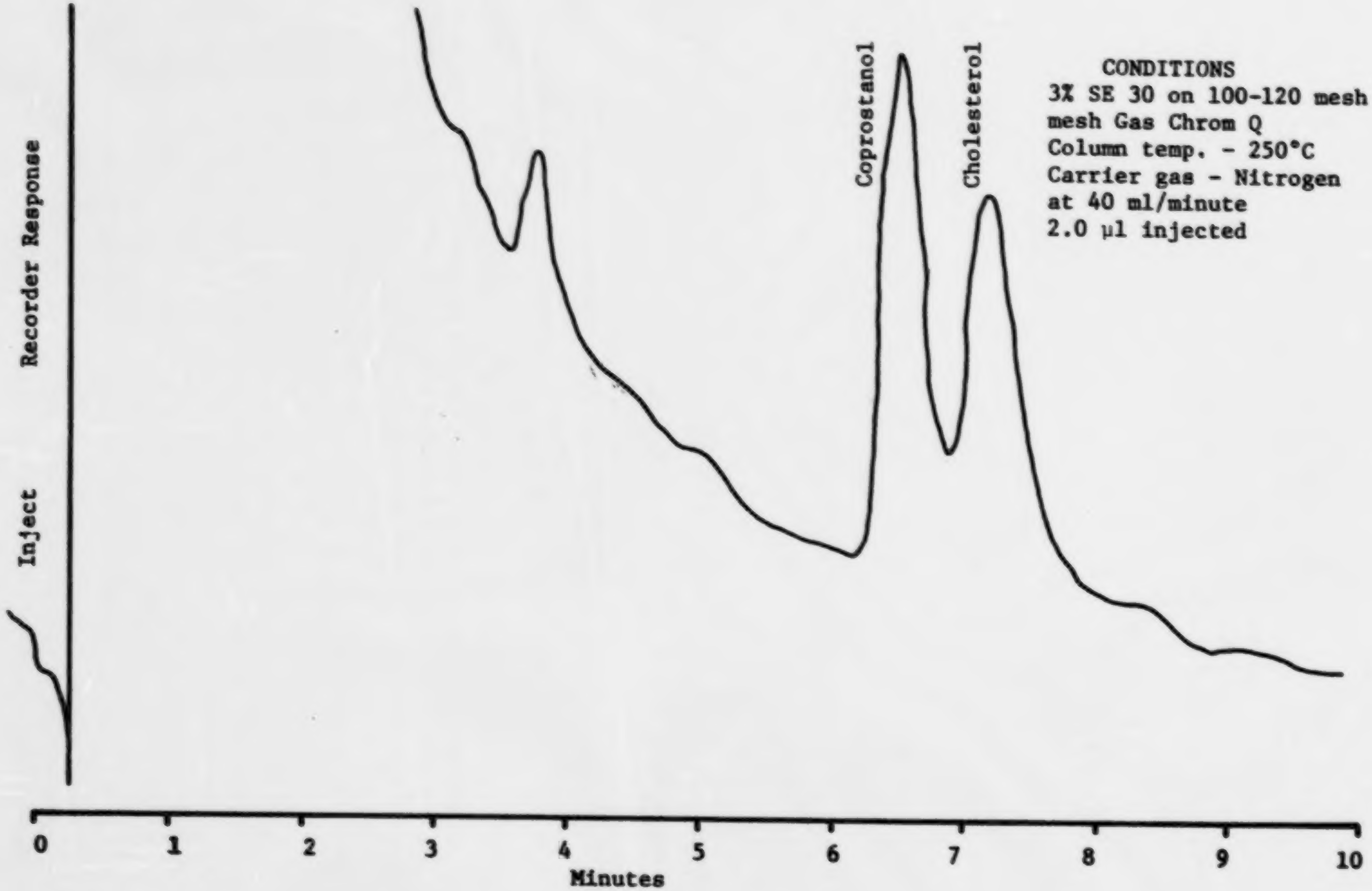


FIGURE 8. CHROMATOGRAM
EXTRACT OF EFFLUENT FROM CITY OF GAINESVILLE TRICKLING FILTER PLANT AFTER SAPONIFICATION

Activated Sludge Effluent

A 10-liter sample was taken from the effluent of the contact stabilization unit of the University Treatment Plant. This sample was divided into one-liter samples, each of which was preserved by the addition of 1 ml concentrated H_2SO_4. Five ml of 20 percent NaCl solution was added to each sample, and the samples were extracted in the usual manner with hexane. The hexane was reduced to dryness, the residue redissolved in 0.5 ml hexane, and chromatographed by GLC. The concentration of coprostanol in each sample was estimated from peak heights. The ten samples were then each dissolved in 50 ml of 7.5 percent KOH in 70 percent ethanol, and then refluxed for three hours.

After this saponification step, each sample was diluted with 50 ml distilled water, and coporstanol was re-extracted with two 25 ml portions of hexane. The hexane was washed with two 15 ml portions of 70 percent ethanol, evaporated to dryness, and the residue redissolved in 0.5 ml hexane. The concentration of coprostanol in these extracts was then determined from the peak heights after gas-liquid chromatography. Results of these analyses are given in Table 2.

TABLE 2

Effect of Saponification on the Analysis of Effluent from the Activated Sludge Process

Sample Number	μg /l Coprostanol Before Saponification	μg/l Coprostanol After Saponification
1	4.55	4.92
2	4.75	5.77
3	4.90	5.15
4	4.30	5.49
5	4.30	5.79
6	4.20	6.18
7	4.20	4.74
8	4.65	5.95
9	4.45	5.49
10	4.25	4.28

Statistical analysis

$Ho \equiv \bar{x}_1 = \bar{x}_2$

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\frac{\sigma_1^2}{n_1} + \frac{\sigma_2^2}{n_2}} = 4.38$$

From standard statistical tables, $t_{\alpha=0.1}=1.734$; result is significant Ho can be rejected and it can be concluded that there is a real difference between the two methods. Calculating the 95 percent confidence interval for sample means we get: Before saponification 4.48 ± 0.14; After saponification 5.38 ± 0.30.

Raw Sewage

The same procedure as in experiment 2 was used except that the source of the sample was raw influent from the University Sewage Treatment Plant. The five-liter sample was divided into ten samples of 500 ml each. Results of these analyses are given in Table 3.

TABLE 3

Effect of Saponification on the Analysis of Raw Sewage Influent

Sample Number	μg/l Coprostanol Before Saponification	μg/l Coprostanol After Saponification
1	554	600
2	522	551
3	576	*
4	520	584
5	548	590
6	536	599
7	522	585
8	504	549
9	502	576
10	492	560

* Sample was lost.

Statistical analysis

$Ho \equiv \bar{x}_1 = \bar{x}_2$ $t = 4.81$

From standard statistical tables, $t_{\alpha=0.1}=1.74$ result is significant, therefore Ho can be rejected and it can be concluded that there is a real difference between the two methods. The 95 percent confidence interval can be calculated: Before saponification 528 ± 15; After saponification 577 ± 12

Trickling Filter Effluent

A ten-liter sample was taken from the trickling filter effluent of the University Sewage Treatment Plant, and then divided into 20 samples of 0.5 liters each. Each sample was preserved with 1 ml of concentrated H_2SO_4 and 3 ml of 20 percent NaCL was added.

Ten samples were extracted in the usual manner. They were not subjected to saponification. After normal extraction the other ten samples were saponified for three hours in 50 ml of 7.5 percent KOH in 70 percent ethanol. The saponified alcohol solutions were diluted with 50 ml of water, followed by a re-extraction with two 25 ml portions of hexane, and washed with two 15 ml portions of 70 percent ethanol.

Both unsaponified and saponified samples were analyzed by GLC. Results of these analyses are given in Table 4.

TABLE 4

Effect of Saponification on the Analysis of Trickling Filter Effluent

Sample Number	µg/l Coprostanol Before Saponification	µg/l Coprostanol After Saponification
1	67.3	67.6
2	65.7	77.8
3	66.9	76.5
4	72.2	82.9
5	84.3	85.9
6	59.9	71.8
7	64.7	82.2
8	69.2	75.9
9	70.2	77.1
10	70.2	67.0
Average	69.1	76.4

Statistical analysis
$H_o \equiv \bar{x}_1 = \bar{x}_2$ $t = 2.61$

From standard statistical tables $t_{\alpha=0.1}=1.734$. Result is significant, therefore, H_o can be rejected and it can be concluded that there is a real difference between the two methods. The 95 percent confidence intervals can be calculated: After saponification 76.40 ± 1.54; Before saponification 69.06 ± 1.46

Precision of Analysis
For those experiments in whichseveral samples were taken for analyses, the precision of the analyses can be calculated.

	Average Error (Expressed as Percent of Mean)	Standard Deviation (Expressed as Percent of Mean)
Raw Sewage		
Before saponification	3.94	4.96
After saponification	2.79	3.40
Trickling Filter		
Before saponification	6.02	9.23
After saponification	6.18	9.93
Activated Sludge		
Before saponification	4.61	4.45
After saponification	9.23	11.2

Stability of Coprostanol toward Microbial Degradation

It has been shown that species of Proactionomyces present in soils are capable of utilizing coprostanol as their sole source of carbon for growth [46]. In addition more than 1,000 bacterial strains are capable of degrading cholesterol which is structurally almost identical to coprostanol [47].

If the disappearance were due to biodegradation, then the rate of disappearance should be affected by chlorination of sewage effluent. In addition, the rate of disappearance of coprostanol is of interest in determining the usefulness of coprostanol as an indicator of fecal pollution.

Smith and Gouron have stated that the removal of coprostanol by the activated sludge process may be due to a physical removal by the floc rather than biodegradation [8].

Laboratory experiments were performed to determine the rate of disappearance of coprostanol in both chlorinated and unchlorinated sewage effluents. At the time microbiological analyses were made to see if the disappearance of coprostanol could be correlated with the decrease in indicator organisms. An experiment of exploratory nature was also performed to determine the mechanism of coprostanol removal in the activated sludge process. Experiments were performed to determine the necessity of preserving samples.

Summary of Results

It is obvious from the degradation studies that coprostanol decreased at a more rapid rate in sewage effluent taken before chlorination than in samples treated by chlorination. One explanation is that the disappearance of coprostanol is due to biological degradation.

The apparent decrease in rate of disappearance of coprostanol with time can be explained as a mass of action effect due to the decreasing concentration of coprostanol with time.

The curve of coprostanol versus time obtained in sample 1 did not fit a first order equation. Two factors may determine the rate change of coprostanol, coprostanol concentration and concentration of organisms capable of biodegrading coprostanol, since both are necessary for the biodegradation to occur.

Results obtained for indicator microorganisms can be divided into two categories, those obtained from chlorinated samples and those obtained from non-chlorinated samples. In general, the results obtained for chlorinated samples were more erratic, showing decreases after chlorination, followed by regrowth of organisms. Non-chlorinated effluents, on the other hand, showed a general decrease in numbers of organisms with time.

Both indicator microorganisms and coprostanol showed the same general trend for non-chlorinated samples, decreasing with time.

Chlorination affected both indicator microorganisms and coprostanol concentration, but in different ways. While indicator microorganisms showed an erattic pattern of decrease followed by regrowth, coprostanol concentration decreased slowly with time. It would appear from these experiments that correlation between indicator microorganisms and coprostanol may be found where the sewage effluent is not chlorinated. Where chlorination is practiced, since the effect of chlorine on microorganisms and coprostanol is different, no correlation may be expected.

Procedure

Four samples of approximately 20 liters each were taken. Samples 1 and 2 were trickling effluent taken from the University Sewage Treatment Plant before chlorination. The third sample was chlorinated effluent from the University Plant and the fourth sample was a 1:2 mixture of chlorinated sewage effluent from the University Plant and a surface water from a small creek entering Payne's Prairie. All samples were taken to the laboratory and placed in large glass cylinders open to the atmosphere, where they were held and stirred constantly during the experiment.

Analyses for coprostanol were made on all samples over a number of days. Simultaneously with coprostanol analyses on samples 2, 3 and 4 total coliform, fecal coliform and standard plate counts were taken. Microbiological tests were also made on the creek water before mixing with sewage effluent. In order to determine if there was any physical removal of coprostanol due to adsorption and deposition onto the glass cylinders, they were washed down with 50-75 ml hexane after termination of the experiments. The hexane was evaporated down to approximately 5 ml and aliquots were analyzed gas chromatographically. Results of the analyses of samples 1-4 are given in Tables 5-8.

Results

No coprostanol was detected in the hexane washings of the glass cylinder after termination of the experiment. Figure 9 shows a plot of the data for change in coprostanol concentration with time found in samples 1 and 2.

Figure 10 shows plots of the rate of change of coprostanol with time as found in samples 3 and 4. Figures 11-13 show the rate of change of microbiological indicators with time as found in sample 2-4.

Activated Sludge--Procedure

One liter of return activated sludge from the University of Florida sewage treatment plant was taken and extracted with two 75 ml portions of

TABLE 5

Data for Sample 1--Trickling Filter Effluent Before Chlorination
(Sample Taken January 26, 1971)

Time (Hours)	Coprostanol (μg/l)
0	95.5
3	78.7
6	86.8
12	76.7
24	47.9
48	21.8
100	11.4
144	5.71
216	5.38
244	4.0

hexane. It was found necessary to add approximately 100 ml, 95 percent ethanol, to break the emulsion that formed. The hexane was separated, washed with two 25 ml portions of ethanol and a gas chromatographic analysis was run on the hexane after reducing the volume to 2 ml by flash evaporation.

TABLE 6

Data for Sample 2--Trickling Filter Effluent Before Chlorination
(Sample Taken February 23, 1971)

Time (Hours)	Coprostanol μg/liter	Total Coliform Number/100 ml	Fecal Coliform Number/100 ml	Standard Plate Counts Number/100 ml
0	70.2	9.7×10^6	4.2×10^5	7.4×10^8
3	55.8	4.7×10^6	2.9×10^5	1.04×10^9
6	67.8	-	-	-
12	54.2	-	-	-
24	32.7	4.2×10^5	3.4×10^4	7.3×10^7
48	17.6	4.8×10^5	2.95×10^4	4.56×10^7
96	-	6.2×10^5	1.41×10^4	5.8×10^7
192	-	4.1×10^5	5.9×10^3	2.15×10^7

No coprostanol was detected in the hexane washings of the glass cylinder after termination of the experiment.

TABLE 7

Data for Sample 3--University Sewage Plant Effluent After Chlorination (Sample Taken February 23, 1971)

Time (Hours)	Coprostanol μg/liter	Total Coliform Number/100 ml	Fecal Coliform Number/100 ml	Standard Plate Counts Number/100 ml
0	42.6	3.2×10^2	2	1.4×10^4
3	39.4	1.7×10^3	9	2.04×10^6
6	41.1	-	-	-
12	40.9	-	-	-
24	47.1	2.08×10^4	0	5.9×10^5
48	35.2	6.6×10^3	2	TNC(>3×10^6)*
96	41.7	TNC(>3×10^6)*	270	1.5×10^8
192	29.7	2.9×10^7	684	4.9×10^8

* TNC--Too Numerous to Count

No coprostanol was detected in the hexane washings of the glass cylinder after termination of the experiment.

TABLE 8

Data for Sample 4--University Sewage Effluent and Surface Water (Sample Taken February 23, 1971)

Time (Hours)	Coprostanol μg/liter	Total Coliform Number/100 ml	Fecal Coliform Number/100 ml	Standard Plate Counts Number/100 ml
0	17.0	1.38×10^4	10	-
3	17.8	2.12×10^5	1300	8×10^5
6	15.0	-	-	-
12	15.7	-	-	-
24	12.2	2.12×10^4	-	1.5×10^6
48	13.9	3.8×10^5	36	TNC(>3×10^6)
96	17.0	2.3×10^7	-	8.9×10^8
192	7.7	3.4×10^6	2	3.5×10^7

Creek Water -	Total Coliform	Fecal Coliform	Standard Plate Count
	1.55×10^3	10	49×10^3

No coprostanol was detected in the hexane washings of the glass cylinder after termination of the experiment.

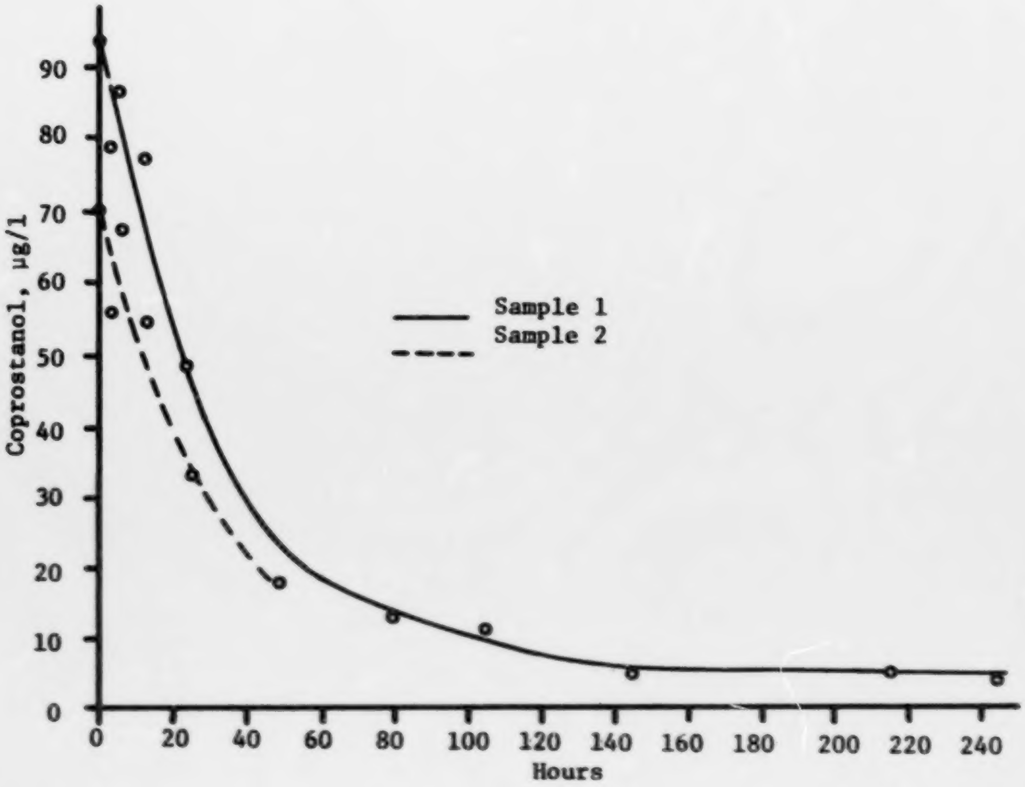


FIGURE 9. RATE OF CHANGE OF COPROSTANOL CONCENTRATION IN SAMPLES 1 AND 2 (TRICKLING FILTER BEFORE CHLORINATION)

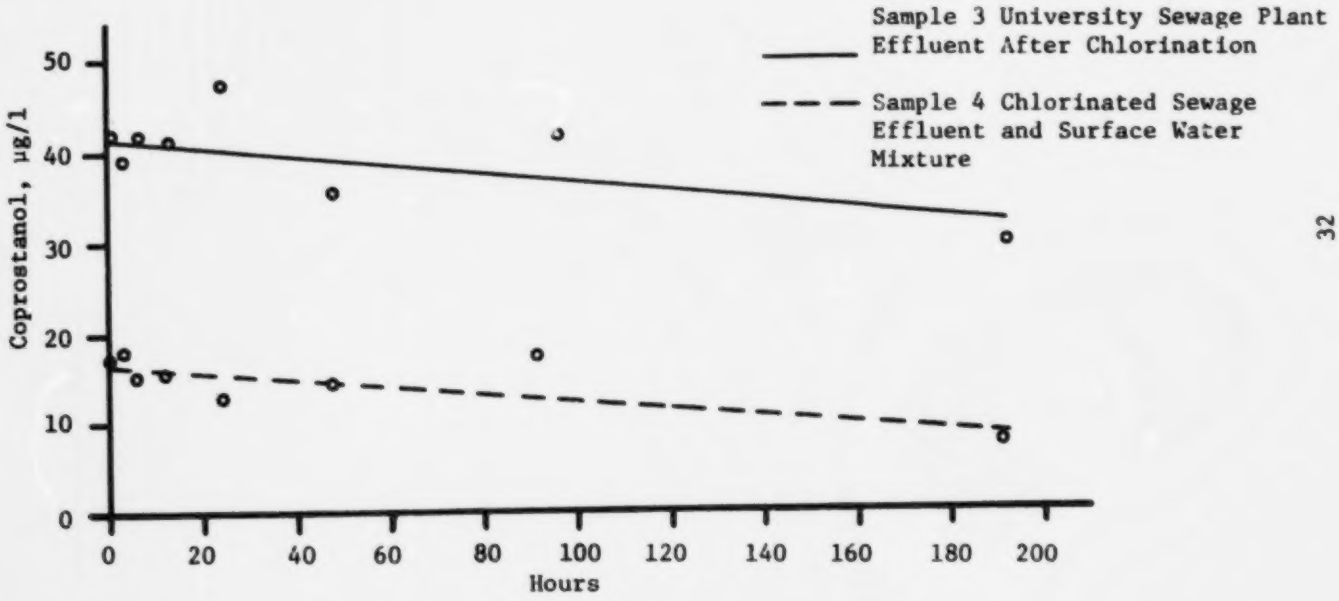


FIGURE 10. RATE OF CHANGE OF COPROSTANOL CONCENTRATION IN SAMPLES 3 **AND** 4

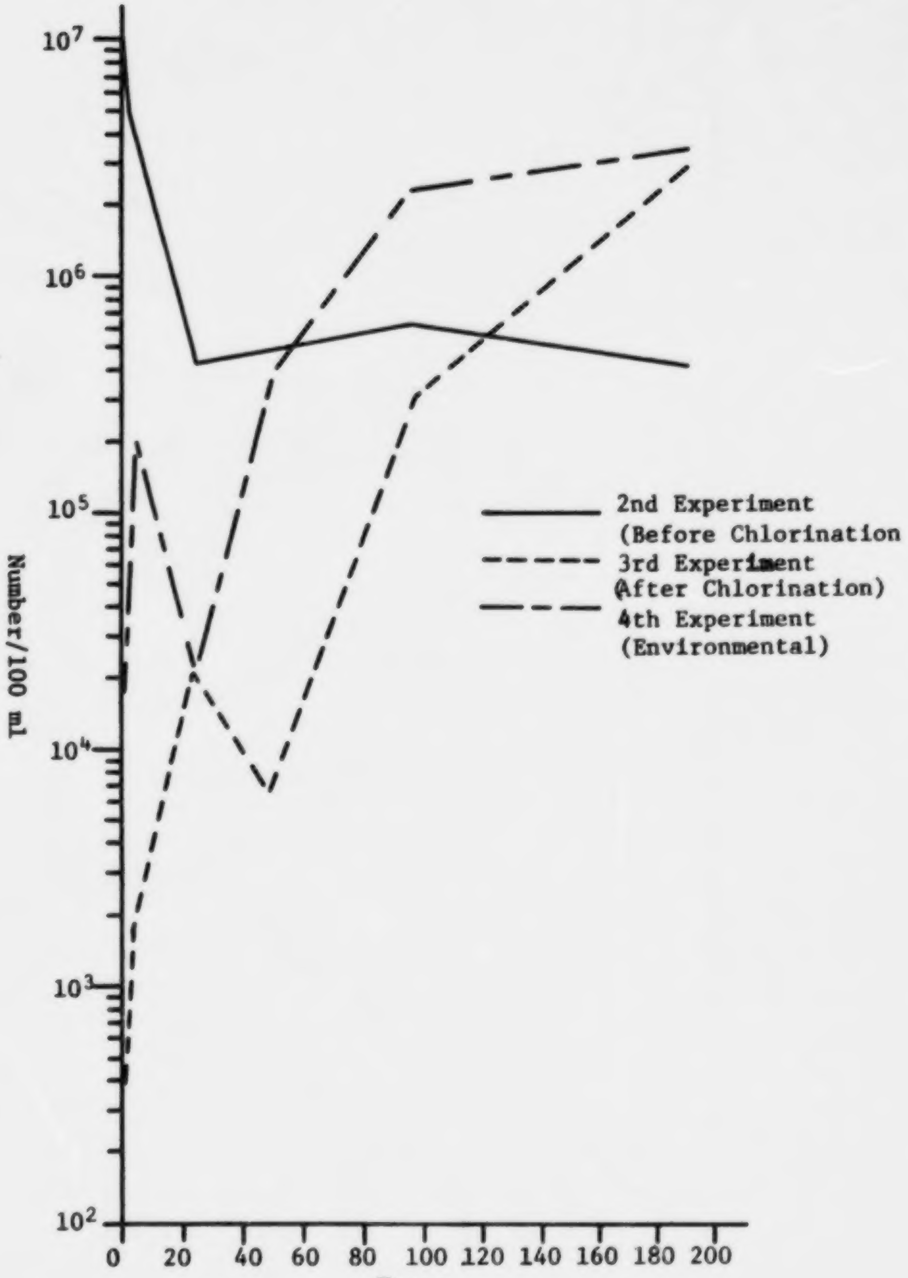


FIGURE 11. TOTAL COLIFORM - RATE OF CHANGE IN CONCENTRATION

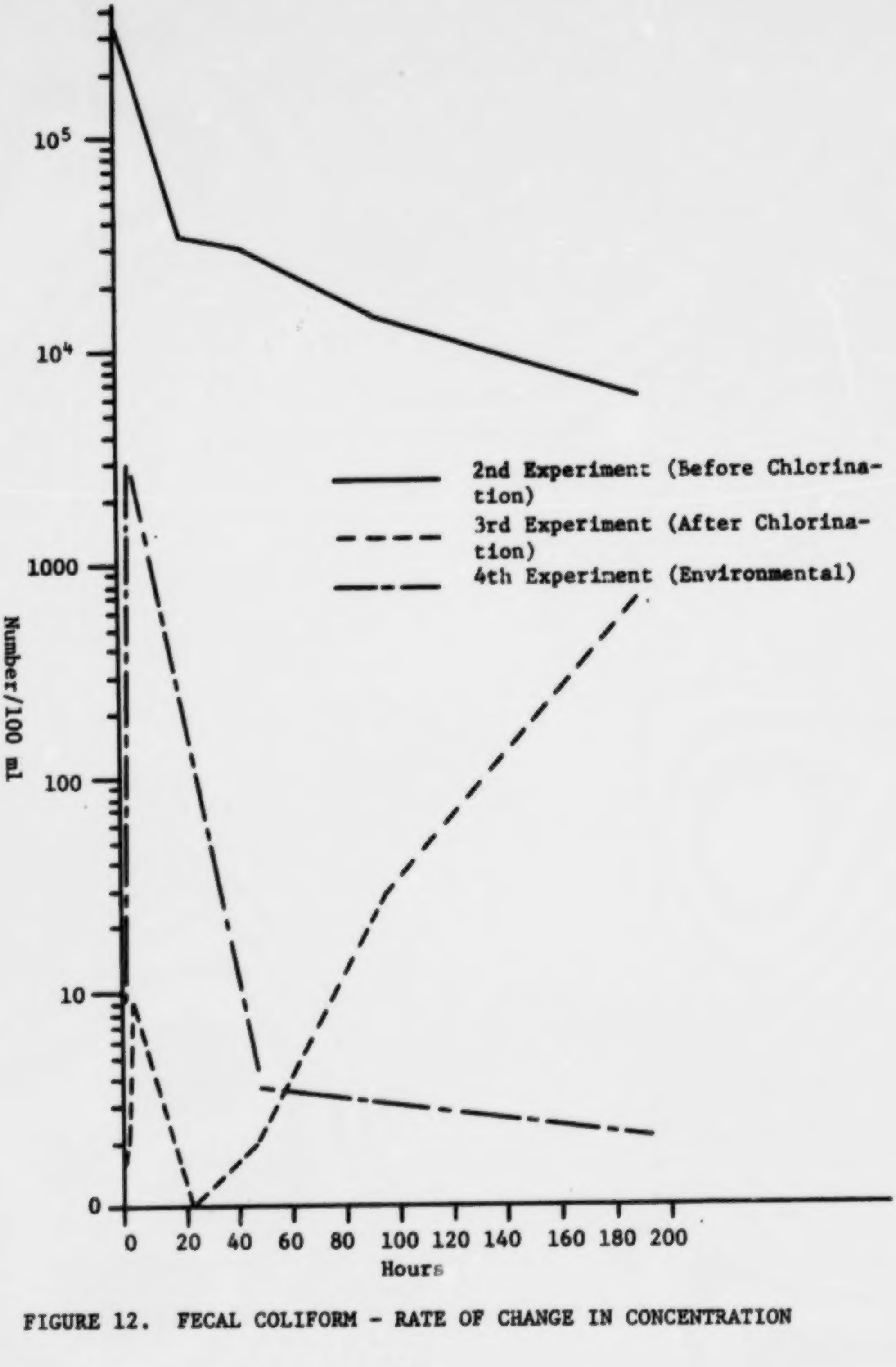


FIGURE 12. FECAL COLIFORM - RATE OF CHANGE IN CONCENTRATION

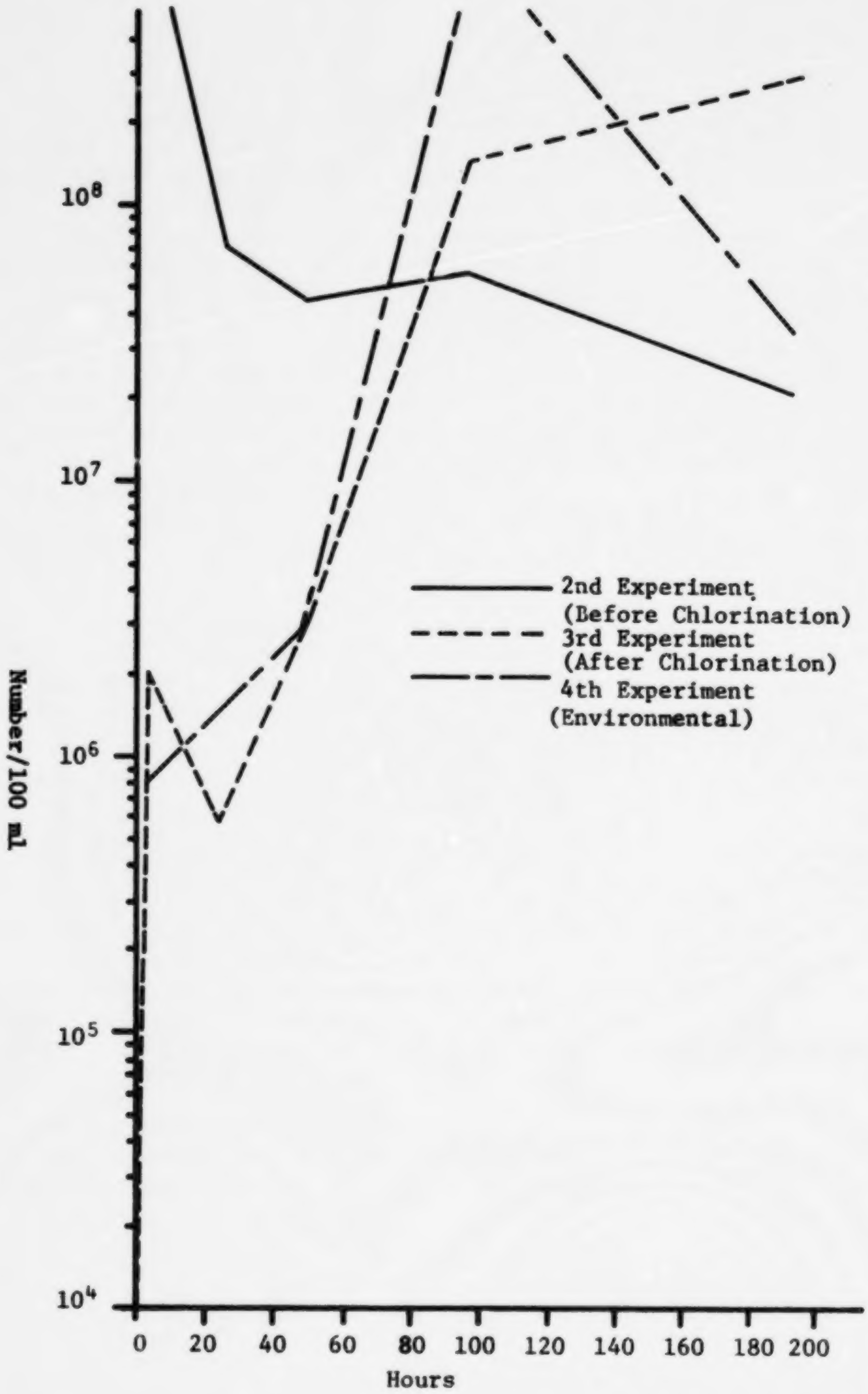


FIGURE 13. 48-HOUR TOTAL PLATE COUNT RATE OF CHANGE IN CONCENTRATION

Results

A very complicated chromatogram was obtained with evidence of coprostanol, as well as cholesterol (Figure 14). Large amounts of cholesterol were found to be present, with lesser amounts of coprostanol. No exact quantitative value for the amount of coprostanol could be determined due to the overlap of another, unknown peak but approximately 100 μg/l of coprostanol was estimated to be present. This value was considerably less than the concentration found in incoming sewage (approximately 500 μg/l) but more than that found in effluent from the activated sludge process (approximately 10 μg/l). Thus it appears that a combination of physical removal and biological degradation may account for the removal of coprostanol, since physical removal would require that coprostanol concentration be higher in the concentrated biological floc than in the sewage influent, while complete biological floc be no higher than that in the effluent.

Preservation of Samples

Summary of Results

Preservation of stored samples is absolutely necessary. The studies show that only 33.4 percent of coprostanol was recovered after 8 days' storage without preservation. The results also indicate that preservation may be accomplished either by the addition of 1 ml of H_2SO_4 per liter of water or by storing the samples at 4°C.

Procedure

A four-liter sample of trickling filter effluent was taken from the Gainesville Sewage Treatment Plant. The sample was divided into eight 500 ml samples. Two samples were preserved with 0.5 ml H_2SO_4 each and analyzed immediately upon return to the laboratory. Two samples were preserved by refrigerating at 4°C and analyzed after 8 days. Two samples were preserved with 0.5 ml H_2SO_4 each and preserved at room temperature. They were analyzed after 8 days in storage. Two samples were stored at room temperature for 8 days and then analyzed. Analysis was done in all cases by extracting with two 50 ml portions of hexane and washing with two 25 ml portions of 70 percent ethanol and two 25 ml portions of acetonitrile saturated with hexane. The hexane extracts were evaporated to dryness and redissolved in 0.5 ml hexane. Five μl were injected for gas chromatography. Results of this experiment are given in Table 9.

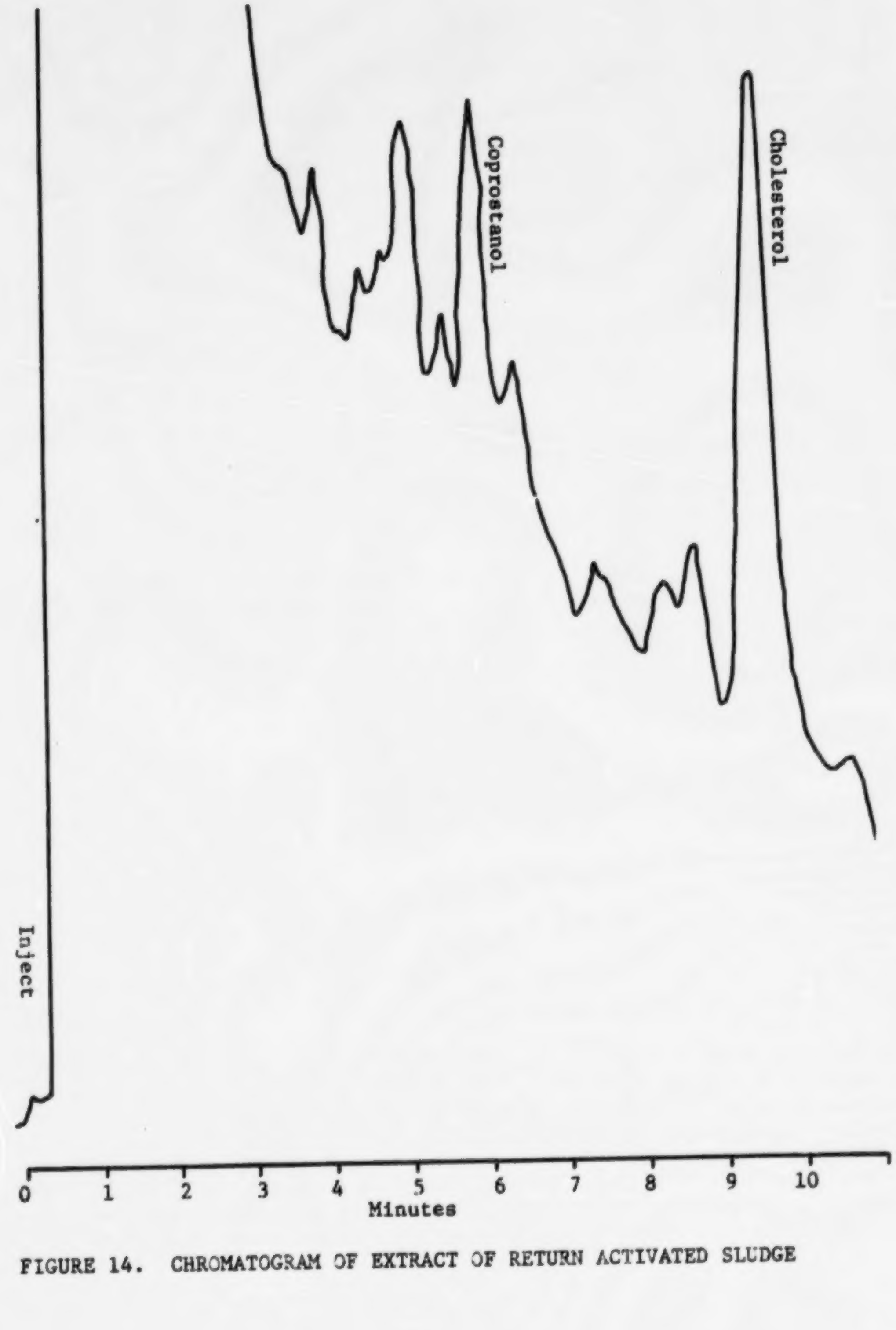


FIGURE 14. CHROMATOGRAM OF EXTRACT OF RETURN ACTIVATED SLUDGE

TABLE 9

Preservation Study Results

Sample	µg/1 Coprostanol
#1 Preserved with H_2SO_4 - No storage	79.4
#2 Preserved with H_2SO_4 - No storage	90.6
#3 Stored at 4°C	73.0
#4 Stored at 4°C	84.0
#5 Preserved with H_2SO_4 - Stored at room temperature	82.0
#6 Preserved with H_2SO_4 - Stored at room temperature	111
#7 Not preserved - Stored at room temperature	30.9
#8 Not preserved - Stored at room temperature	26.0

Assuming the average value of samples #1 and #2 represents the true value of coprostanol concentration in the original sample, we can calculate recoveries for the various experiments.

Preserved by refrigeration

$$\%\ \text{Recovery} = \frac{78.5}{85.0} \times 100 = 92.6\%$$

Preserved by addition of H_2SO_4

$$\%\ \text{Recovery} = \frac{96.5}{85.0} \times 100 = 113\%$$

No preservation

$$\%\ \text{Recovery} = \frac{28.4}{85.0} \times 100 = 33.4\%$$

Field Surveys

The field studies reported here were undertaken in order to determine the applicability of coprostanol as a useful indicator of water quality, and to determine any possible relationship between coprostanol and biological indicators in water samples.

Surveys were made of two areas in the city of Gainesville, Florida. The first area surveyed was Sweetwater Creek--Payne's Prairie, and the second was the University of Florida Sewage Treatment Plant--Lake Alice.

Samples were taken at locations shown on the maps (Figures 15 and 16). Numbers on the maps correspond to station numbers in Tables 10 and 11.

Summary of Results

The concentration of coprostanol decreased with distance from the sewage plant outfall but did not correlate with total or fecal coliform analyses because of the effect of chlorination on coliforms and because of a large bird population in Payne's Prairie. Samples taken from Sweetwater Creek above the sewage plant outfall had very low coprostanol concentrations.

Every sample for chemical analyses was preserved with 1 ml conc. H_2SO_4 per liter. Extraction with hexane was done as soon as possible after sampling. Extraction and analysis were done in the manner detailed previously. None of the extracts were saponified to free the esters. It was found necessary to clean up by thin-layer chromatography all samples with a coprostanol contant less than 1 μg/l. Thin-layer chromatography was performed using Eastman Chromagram Sheets. Development of the plates was with benzene.

Standard coprostanol and cholesterol were identified by spraying with a saturated solution of antimony trichloride and chloroform, as well as with ultraviolet light. Unknown locations corresponding to those of standards were cut out using scissors and redissolved in a small amount of hexane. Samples for microbiological tests were also taken at the same time as the chemical samples, and were analyzed the same day for total coliform, fecal coliform and standard plate count.

The concentration of coprostanol in Sweetwater Creek and Payne's Prairie was found to decrease with increase in distance from the source of pollution, the City of Gainesville Sewage Treatment Plant.

High concentrations of coprostanol were found in all samples taken in Sweetwater Creek downstream from the treatment plant. Very low concentrations of coprostanol were found in samples taken upstream from the treatment plant as well as for samples taken well inside Payne's Prairie.

No apparent relationship between coprostanol and any of the microbiological indicators could be found. Chlorination had the effect of lowering appreciably the concentration of microorganisms, without apparently affecting the coprostanol concentration. The result was that low counts of microorganisms were found near the plant, where the water was hardly of good quality, while high counts of microorganisms were found at distant points where one would have expected water quality to have been improved. High counts of coliform organisms found in Payne's Prairie may have been due to the very high aquatic bird population (estimated 15,000-20,000 ducks, 1,000 Sand Hill Cranes and 3,000 egrets and herons) [48] found there during the winter months. One duck is said to contribute 11,000 Fecal Coliform per 24 hours

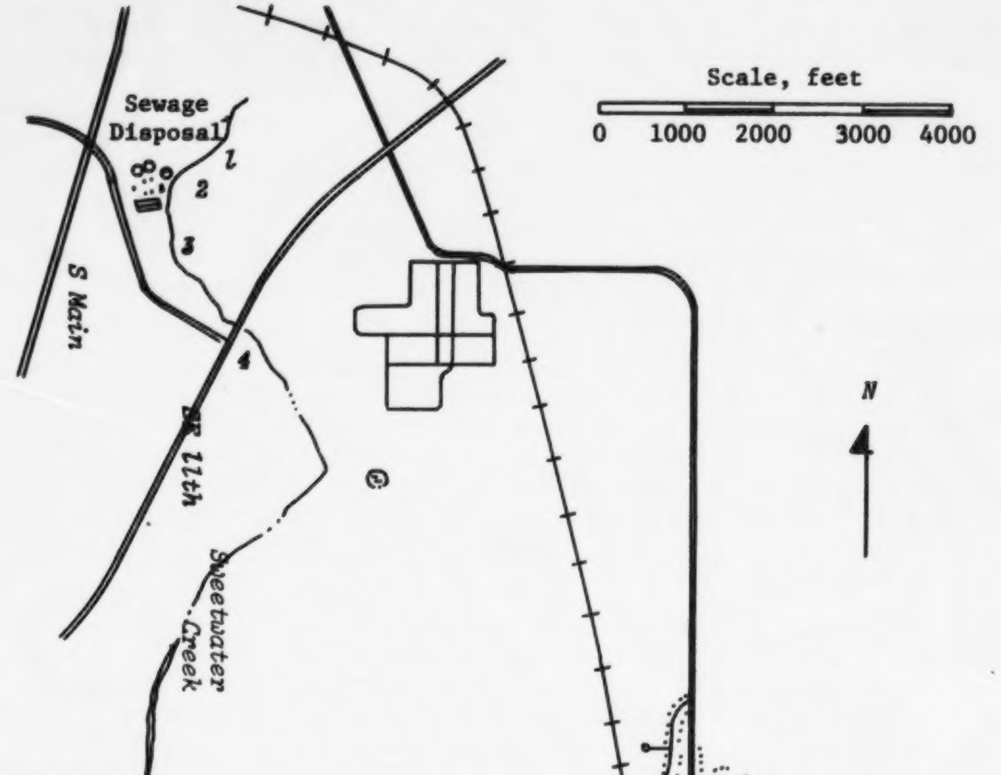

Scale, feet
0
1000
2000
3000
4000
Sewage Disposal
1
2
3
4
S Main
SW 11th
Sweetwater Creek
N
5
6
7
8
9
Payne's Prairie
Alachua Sink

FIGURE 15. SAMPLING STATIONS, SWEETWATER CREEK-PAYNE'S PRAIRIE

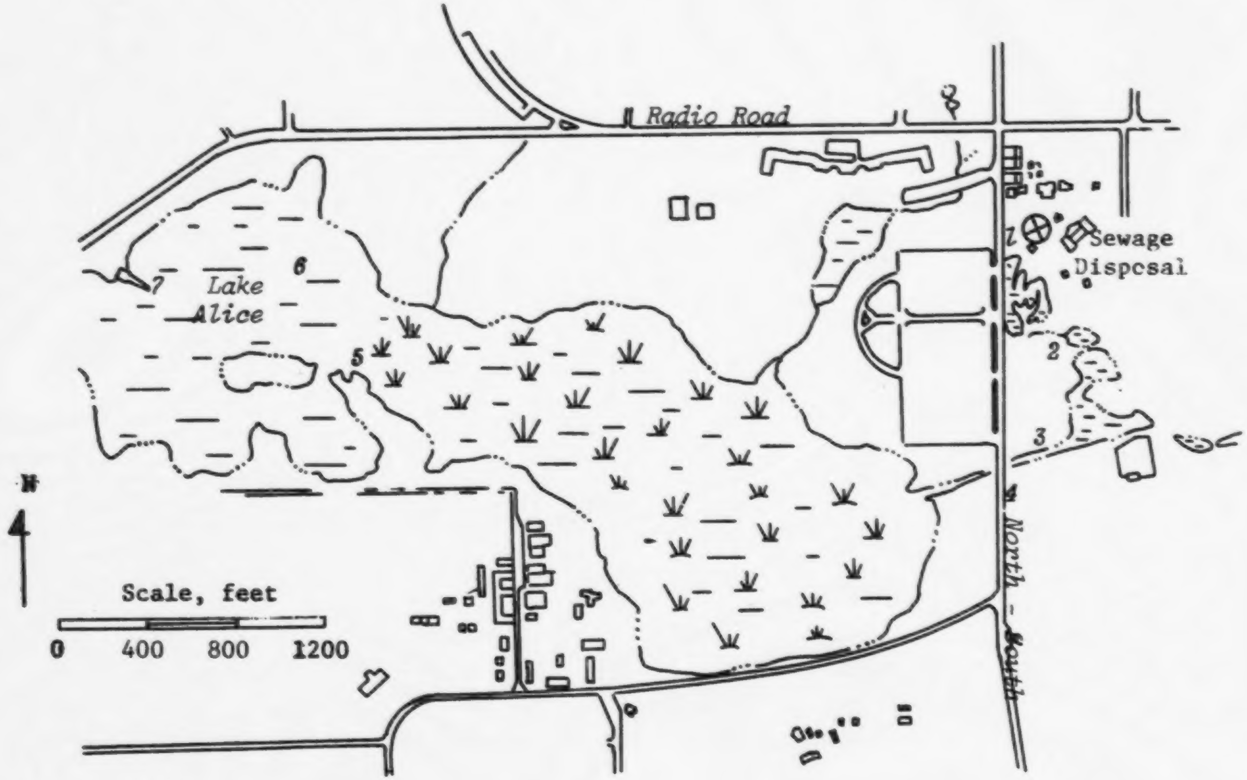

Radio Road
Lake Alice
Sewage Disposal
North - South
1
2
3
4
5
6
7
N
Scale, feet
0
400
800
1200

FIGURE 16. SAMPLING STATIONS, UNIVERSITY OF FLORIDA SEWAGE TREATMENT PLANT LAKE ALICE

TABLE 10

Survey for Sweetwater Creek--Payne's Prairie (March 9, 1971)

Station	Location	Volume of Sample, liters	Coprostanol µg/l	Total Coliform Number/100 ml	Fecal Coliform Number/100 ml	48 Hr. Standard Plate Count Number/100 ml
1	100 feet upstream from sewage outfall	1.0	0.027	6.4×10^4	80	3.8×10^6
2	Sewage effluent	0.5	65.0	26	0	7.4×10^3
3	300 feet downstream from sewage outfall	0.5	50.9	12	1	6.2×10^3
4	Highway 331 and Sweetwater Creek	1.0	39.2	72	0	2.8×10^4
5	Termination of Sweetwater Creek	1.0	24.3	1.12×10^3	0	1.12×10^6
6	120 feet inside Payne's Prairie	1.0	24.9	3.8×10^2	0	1.03×10^7
7	Intersection of North-South and East-West Canals	1.0	0.033	1.47×10^4	61	6.9×10^6
8	250 yards south of Station 7	1.0	0.059	1.78×10^4	57	4.95×10^6
9	Alachua Sinkhole	1.0	0.061	3.38×10^4	15	3.35×10^6

TABLE 11

Survey Data for University of Florida Sewage Treatment Plant--Lake Alice
(March 23, 1971)

Station	Location	Volume of Sample, liters	Coprostanol μg/l	Total Coliform Number/100 ml	Fecal Coliform Number/100 ml	48 Hr. Standard Plate Count Number/100 ml
1	Effluent after chlorination	0.5	54	3.22×10^4	9	1.87×10^7
2	After oxidation pond	0.5	15	5.42×10^5	960	1.06×10^7
3	100 yards downstream from #2	1.0	9.9	5.55×10^5	1.37×10^3	4.0×10^7
4	Near underpass of canals in North-South Road	1.0	2.4	1.85×10^5	250	2.03×10^7
5	East end of Lake Alice	1.5	0.22	7.3×10^4	-	1.68×10^8
6	Middle of Lake Alice	1.5	0.014	1.37×10^4	96	1.46×10^8
7	West end of Lake Alice near out flow	1.5	0.022	1.17×10^2	66	1.34×10^7

while a man contributes 2,000 Fecal Coliform [49]. Thus, a 15,000-20,000 duck population alone would correspond to the Fecal Coliform production of a human population of 82,500 to 110,000.

As in the first survey, the concentration of coprostanol in the University of Florida Sewage Treatment Plant--Lake Alice survey was found to decrease with increasing distance from the source of pollution. There was a significant decrease in coprostanol as the sewage effluent passed through the oxidation pond (54 µg/l to 15 µg/l). Fairly high concentrations of coprostanol were found in all samples taken from the stream leading into Lake Alice. However, very low concentrations of coprostanol were found in Lake Alice itself. Only the east end of Lake Alice could be said to contain significant amounts of coprostanol. No difficulty was encountered in analyzing for free coprostanol in the environment.

Once again chlorination had the effect of decreasing significantly the number of microorganisms to be found in the water in samples taken near the sewage outfall. There was an apparent regrowth followed by a decrease in numbers of coliform organisms. This effect of regrowth is apparently a common phenomenon. As a result of the changing character of the coliform organisms, no relationship could be found between these microbiological indicators and coprostanol levels.

It would appear from the above study that no simple relationship bewteen microbiological indicator organisms and coprostanol exists, especially if the sewage effluent is highly chlorinated. Coprostanol would appear to be a better indicator of fecal pollution than coliform in those waters which receive highly chlorinated effluent. Coprostanol concentrations in such cases would be high, where coliform organisms may be quite low and a danger to either resistant pathogenic bacteria or viruses may be present.

High aquatic bird populations may cause high coliform counts in water [49], while no real danger exists. Although some diseases may be transmitted from birds to man [50], none of these diseases are normally transmitted by the water route. None of the major water borne diseases listed in *Control of Communicable Diseases in Man* [51] are given as being caused by birds as reservoirs of the disease.

CORRELATION OF COPROSTANOL CONCENTRATION WITH OTHER MEASURES OF DOMESTIC WASTEWATER TREATMENT PLANT EFFICIENCY

There is a growing need for evaluation of wastewater treatment plant efficiency in order to improve design and operation of such plants as well as to improve the quality of receiving waters.

Presently, the biochemical oxygen demand, BOD, test has the widest application in measuring waste loadings to treatment plants and in evaluating the treatment efficiency. The test is extremely time consuming. Comparison of BOD values cannot be made unless the results have been obtained under identical test conditions. Another popular parameter used is COD (chemical oxygen demand). The method needs close control and fails to include some organic compounds which are biologically available to the organisms. Unpolluted waters may have anomolously high BOD and COD values. Both tests suffer from the fact that they are neither specific nor precise.

A specific indicator which could be used is coprostanol. As reported earlier, coprostanol loading has been estimated to be approximately 2 grams per capita per day [5]. Coprostanol was found to occur at detectable levels in sewage treatment effluents and is removable by adequate secondary sewage treatment [5]. Investigations on the Missouri River showed that coprostanol level varied with the degree of domestic waste pollution [52].

It was the purpose of this phase of the study to determine the possibility of using coprostanol as an indicator of domestic waste treatment plant efficiency. Efforts were made to correlate coprostanol with BOD and COD as well as TOC (total organic carbon). Evaluation and control of domestic wastewater treatment efficiency could be greatly facilitated if such relationships could be found.

Summary of Results

There was a good correlation between TOC and coprostanol concentration with a coefficient of determination (R^2) of 0.977 for a linear fit. COD and coprostanol could be related by a parabolic curve but R^2 is only 0.630. BOD and coprostanol could be related linearly but R^2 is even lower, only 0.429.

Many more data points are needed to confirm these relationships.

Procedure

The University of Florida Campus Sewage Treatment Pland and Gainesville Municipal Sewage Treatment Plant (both in Gainesville, Florida) were selected for sampling sites due to their domestic wastewater source, close control and availability. Samples were collected at different times from different locations in order to obtain a wide range of variations of the wastewater characteristics. In order to obtain representative wastewater samples and to have maximum accuracy on analysis, three-hour composite sampling was adopted. BOD, COD and TOC analyses were performed immediately after sampling to prevent preservation error. Coprostanol samples were put in one-liter glass bottles and preserved by acidification (3 ml concentrated sulfuric acid per liter sample) and refrigeration. Analysis was done within 5 hours after sampling.

BOD--The analysis was done according to *Standard Methods for the Examination of Water and Wastewater* [1]. Three dilutions were made of each sample. Contact stabilization plant raw influent was used for seeding material. *Modified Winkler Method (Azide Modification) with Full-Bottle Technique* developed by the Environmental Protection Agency was used for the dissolved oxygen measurements [53].

COD--The dichromate reflux method described in *Standard Methods* [1] was used for high level COD analysis. Low level COD analysis was carried out following procedures outlined in the *EPA Manual* [53].

TOC--Samples for TOC analysis were put in 150 ml glass bottles to prevent the interference of extractable carbonaceous substances. Precautions were taken to avoid decomposition from exposure to light. Analyses were performed immediately after sampling on the Dow-Beckman Carbonaceous Analyzer Model 915.

Coprostanol--Samples were analyzed by GLC as discussed earlier.

Results

Eight samples were taken from primary treatment step of the University of Florida Campus Sewage Treatment Plant (the results are shown in Table 12), 22 samples from the trickling filter plant (Table 13), 18 samples from the contact stabilization plant (Table 14), and 6 samples from the tertiary treatment (aerated lagoon, Table 15). Ten samples were taken from the contact stabilization plant of the Gainesville Municipal Sewage Plant (Table 16) and 7 samples from trickling filter plant Table 17). For the special experiment on TOC analysis, 10 samples were taken from the municipal contact stabilization plant (Table 18).

In order to confirm the possible relationships among coprostanol and BOD, COD and TOC, the authors tried to fit the data to equations. The data were plotted. By comparison with figures of various functions, the linear form of polynomial functions was chosen as the model. A general form of the model can be represented by: $Y = \beta_o + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \ldots + \beta_kX_k + \varepsilon$, where Y is the dependent variable relating to a set of independent variables, X represents unknown parameters, ε is the random error. No interaction terms were included and the second order terms were the highest order used.

A Linear Least Square Curve Fitting Program was used to determine whether the equation chosen could be made to fit the data by a suitable choice of coefficients. By computer analysis with APL (A Programming Language) on an IBM 360 computer 2741 terminal, the following major information was obtained on each set of data [54]:
1. Coefficient of determination (R^2), i.e., how well the equation fitted. If $R^2 = 0.700$ for example, it means that 70 percent of the

TABLE 12

DATA FROM CAMPUS PLANT-PRIMARY TREATMENT

Sample No.	Sampling Station	BOD (mg/l)	COD (mg/l)	Coprostanol (µg/l)
1	P.I.	267.50	364.14	200.00
2	P.E.	47.50	77.60	10.00
3	P.I.	62.50	85.36	13.75
4	P.E.	100.00	234.74	50.00
5	P.I.	137.50	122.00	36.25
6	P.E.	120.00	135.00	41.66
7	P.I.	245.00	263.00	82.50
8	P.E.	182.50	213.00	92.50

P.I.: Primary Influent
P.E.: Primary Effluent

TABLE 13

DATA FROM CAMPUS TRICKLING FILTER PLANT

Sample No.	Sampling Station	BOD (mg/1)	COD (mg/1)	Coprostanol (μg/1)
1	TF-R	267.50	364.14	200.00
2	P-1	106.00	180.14	76.00
3	TF-1	35.03	128.36	35.00
4	S-1	29.50	103.46	29.00
5	TF-1	8.13	52.38	65.00
6	S-1	20.50	73.72	56.25
7	TF-R	47.50	77.60	10.00
8	S-1	16.25	42.68	3.33
9	TF-1	3.60	8.00	3.33
10	P-1	26.25	27.16	43.75
11	S-4	*	83.42	6.66
12	S-3	*	60.14	12.50
13	S-1	*	79.54	71.25
14	TF-1	*	87.30	6.25
15	TF-2	*	56.26	56.25
16	P-4	*	230.00	110.00
17	P-1	*	217.28	40.00
18	TF-R	*	234.74	50.00
19	TF-R	137.50	122.00	36.25
20	TF-R	120.00	135.00	41.66
21	TF-R	245.00	263.00	82.50
22	TF-R	182.50	213.00	92.50

TF-R: Raw Influent
P-1: Undiluted Primary Clarifier Effluent
P-4: Diluted Primary Clarifier Effluent
TF-1: High Rate Trickling Filter Effluent
TF-2: Standard Rate Trickling Filter Effluent
S-1: No. 1 Secondary Clarifier Effluent
S-3: No. 2 Secondary Clarifier Effluent
S-4: Combined Effluent of Secondary Clarifier
*Data unavailable due to missing BOD bottles

TABLE 14

DATA FROM CAMPUS CONTACT STABILIZATION PLANT

Sample No.	Sampling Station	BOD (mg/l)	COD (mg/l)	Coprostanol (μg/l)
1	CSP2	28.25	295.00	141.00
2	CSP1	53.00	201.00	33.75
3	CSPR	237.50	380.45	162.00
4	CSPR	46.66	407.59	140.00
5	CSPR	105.00	337.93	142.50
6	CSPR	24.16	409.21	159.00
7	CSPR	43.33	72.35	5.00
8	CSPR	19.16	72.25	4.50
9	CSP2	14.00	41.47	3.00
10	CSP2	7.00	400.00	10.50
11	CSP2	10.00	180.00	25.00
12	CSP2	3.00	104.75	3.50
13	CSP2	4.00	36.70	1.66
14	CSP2	5.50	33.37	0.10
15	CCE	*	0.10	1.00
16	CSPR	*	153.26	67.50
17	CSP2	*	64.02	12.50
18	CSP1	*	40.74	1.75

CSPR: Contact Stabilization Plant Raw Influent
CSP1: Effluent from Contact Plant No. 1
CSP2: Effluent from Contact Plant No. 2
CCE: Combined Effluent of Contact Plant No. 1 and No. 2
*Data unavailable due to missing BOD bottles

TABLE 15

DATA FROM CAMPUS TERTIARY TREATMENT

Sample No.	Sampling Station	BOD (mg/1)	COD (mg/1)	Coprostanol (μg/1)
1	L.I.	21.00	19.00	3.93
2	L.E.	19.00	7.60	5.00
3	L.I.	16.00	34.30	1.88
4	L.E.	21.00	19.00	4.17
5	L.I.	6.00	27.16	18.75
6	L.E.	6.50	42.88	0.17

L.I.: Lagoon Influent
L.E.: Lagoon Effluent

TABLE 16

DATA FROM MUNICIPAL CONTACT STABILIZATION PLANT

Sample No.	Sampling Station	BOD (mg/1)	COD (mg/1)	Coprostanol (μg/1)
1	A	263.25	300.00	115.00
2	C	56.00	7.60	0.01
3	D	8.00	7.60	0.01
4	E	6.67	5.32	13.75
5	B	220.00	231.00	33.33
6	A	167.50	427.00	100.00
7	C	38.00	49.40	15.00
8	E	8.15	22.80	0.62
9	D	4.46	38.80	3.33
10	B	145.00	53.20	80.00

A: Contact Plant Raw Influent
E: Primary Treatment Effluent
B: Contact Basin Effluent
C: Clarifier Effluent
D: Final Effluent of Municipal Plant

TABLE 17

DATA FROM MUNICIPAL TRICKLING FILTER PLANT

Sample No.	Sampling Station	BOD (mg/l)	COD (mg/l)	Coprostanol (μg/l)
1	G	110.00	133.00	53.33
2	H	30.00	40.00	14.00
3	K	16.00	10.25	54.16
4	G	96.50	202.00	133.75
5	H	29.00	86.50	31.66
6	K	112.50	108.00	177.50
7	F	145.00	407.00	116.66

K: Trickling Filter Raw Influent
F: Primary Clarifier Influent
G: Trickling Filter Effluent
H: Secondary Clarifier Effluent

TABLE 18

DATA FROM THE SPECIAL STUDY ON THE MUNICIPAL CONTACT STABILIZATION PLANT

Sample No.	Sampling Station	BOD (mg/l)	COD (mg/l)	TOC (mg/l)	Coprostanol (μg/l)
1	A	121.00	255.36	126.00	48.00
2*	B	---	---	---	--
3	C	10.00	17.28	12.00	11.07
4	D	2.87	5.76	12.00	11.43
5*	E	---	---	---	--
6	F	120.00	211.20	8.70	10.00
7	G	12.50	53.76	8.00	3.76
8	H	5.00	92.16	6.00	5.83
9	I	2.00	34.56	2.50	6.43
10	J	2.40	80.64	2.50	4.06

*Results on Sample No. 2 and No. 5 were discarded due to extremely high TOC values compared to low values of BOD, COD and coprostanol

sum squared of deviations of the Yi about their mean can be accounted for by the variables X_1, X_2 ... X_k.
2. The magnitudes of the intercept (β_o) and of the other coefficients (β_i's).

Precision--For each method, one sample was portioned into ten equal parts and analyzed simultaneously. The results are shown in Table 19.

TABLE 19

Precision Test Results

Test	Mean Value	Standard Deviation	Relative Standard Deviation with Respect to Mean
Coprostanol	57.50 μg/1	0.72 μg/1	1.25%
COD	137.50 mg/1	3.00 mg/1	2.18%
BOD	82.50 mg/1	4.28 mg/1	5.18%
TOC	*	*	5-10%**

*Unavailable
**Data from Standard Methods, 13th edition

Statistical Analysis of Results

An example of the technique used for analysis of the results of a given set of data is shown below for Experiment 1 - Campus Plant--Primary Treatment. Model I in each case was a parabolic function and Model II linear. The curves of best fit are shown in Figures 17-21 and a summary of the statistical results are shown in Table 20.

Experiment 1 - Campus Plant--Primary Treatment

a. Coprostanol vs. COD

Y: Coprostanol
X: COD

Model I: $Y = \beta_o + \beta_1 X + \beta_2 X^2 + \varepsilon$

Prediction equation: $y = 23.5 - 0.197X + 0.00182X^2$ (1)

$n = 8$ $R^2 = 0.878$

Test on Curvature by two-sided student's t test:

H_o: $\beta_2 = 0$

TS: $t = \frac{\hat{\beta}_2 - 0}{MSEC_{22}} = 2.02$

RR: $|t| > \frac{t\alpha}{2}$, d.f.

From standard statistical tables,

$t_{0.025,5} = 2.57$ $t_{0.05,5} = 2.01$

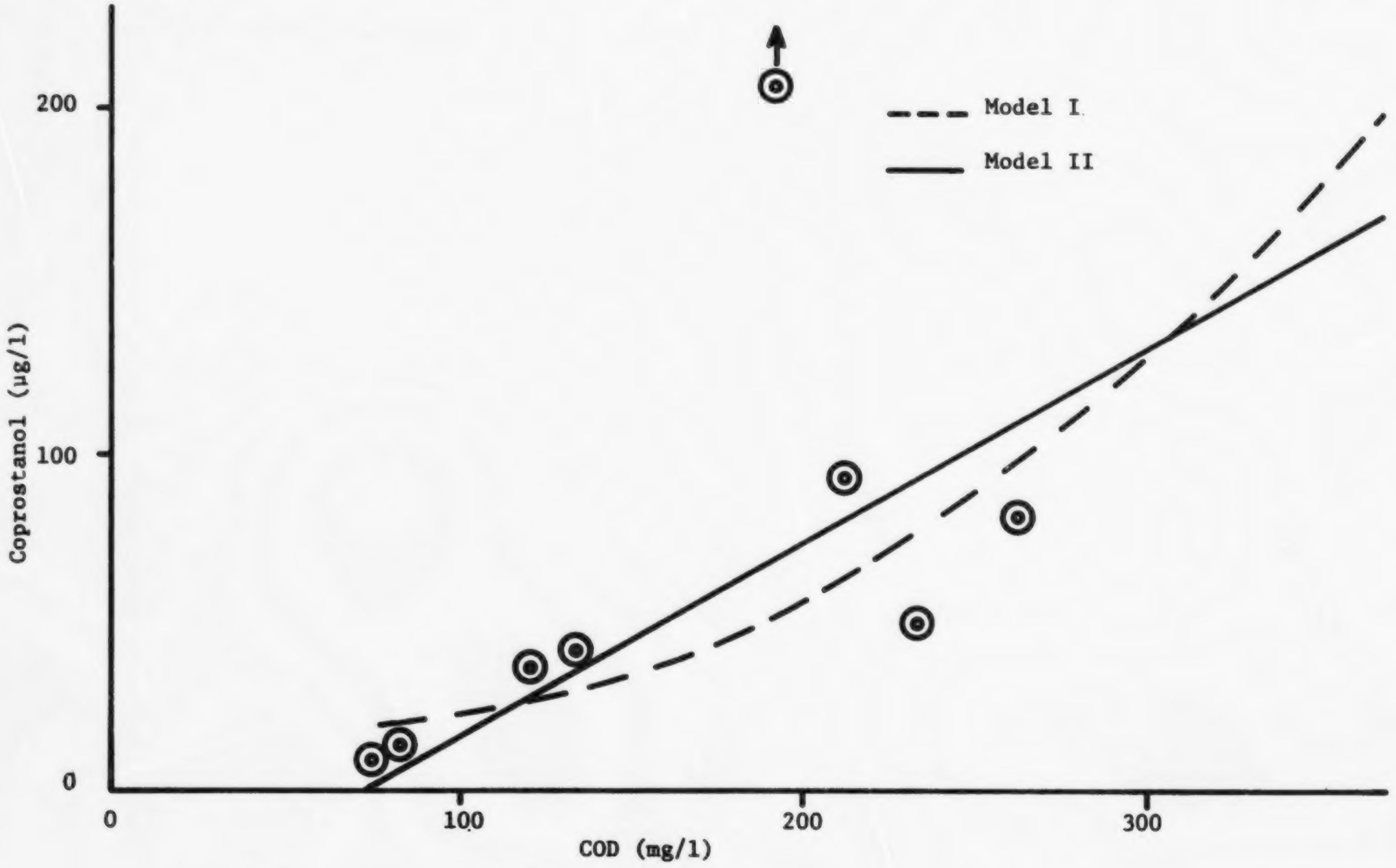


FIGURE 17. COPROSTANOL vs COD CAMPUS PLANT--PRIMARY TREATMENT

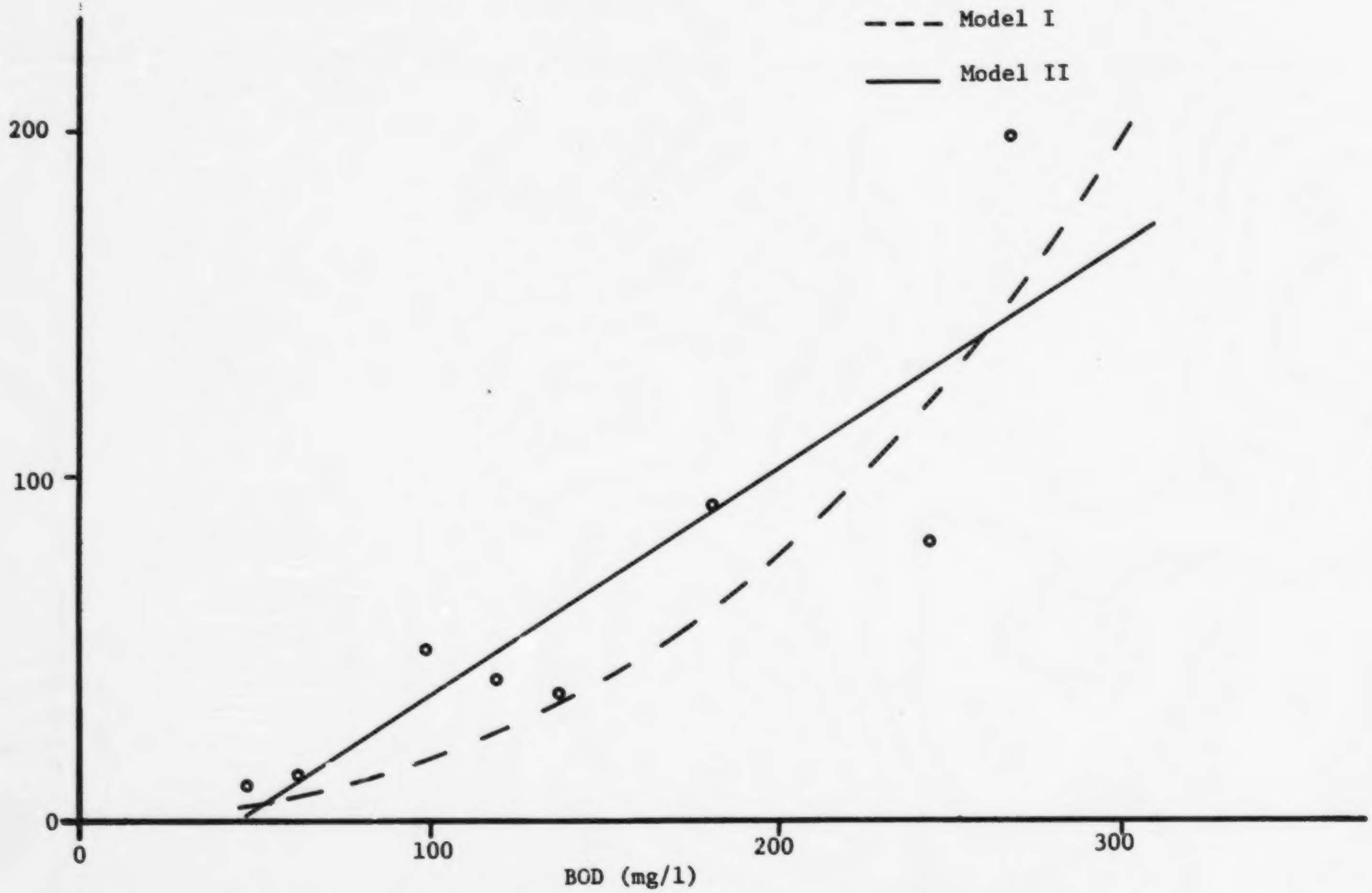


FIGURE 18. COPROSTANOL vs BOD CAMPUS PLANT--PRIMARY TREATMENT

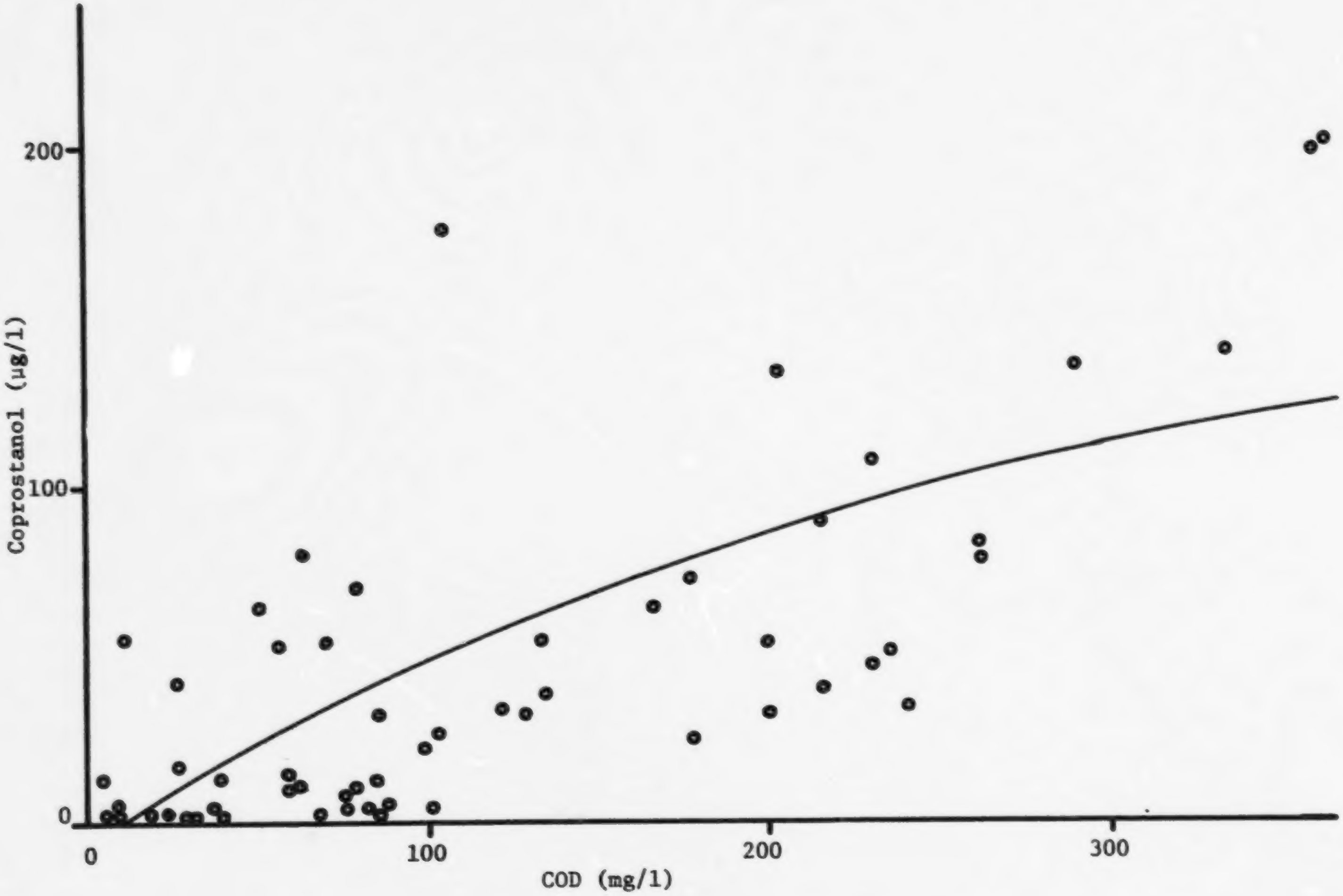


FIGURE 19. COPROSTANOL vs COD OVERALL

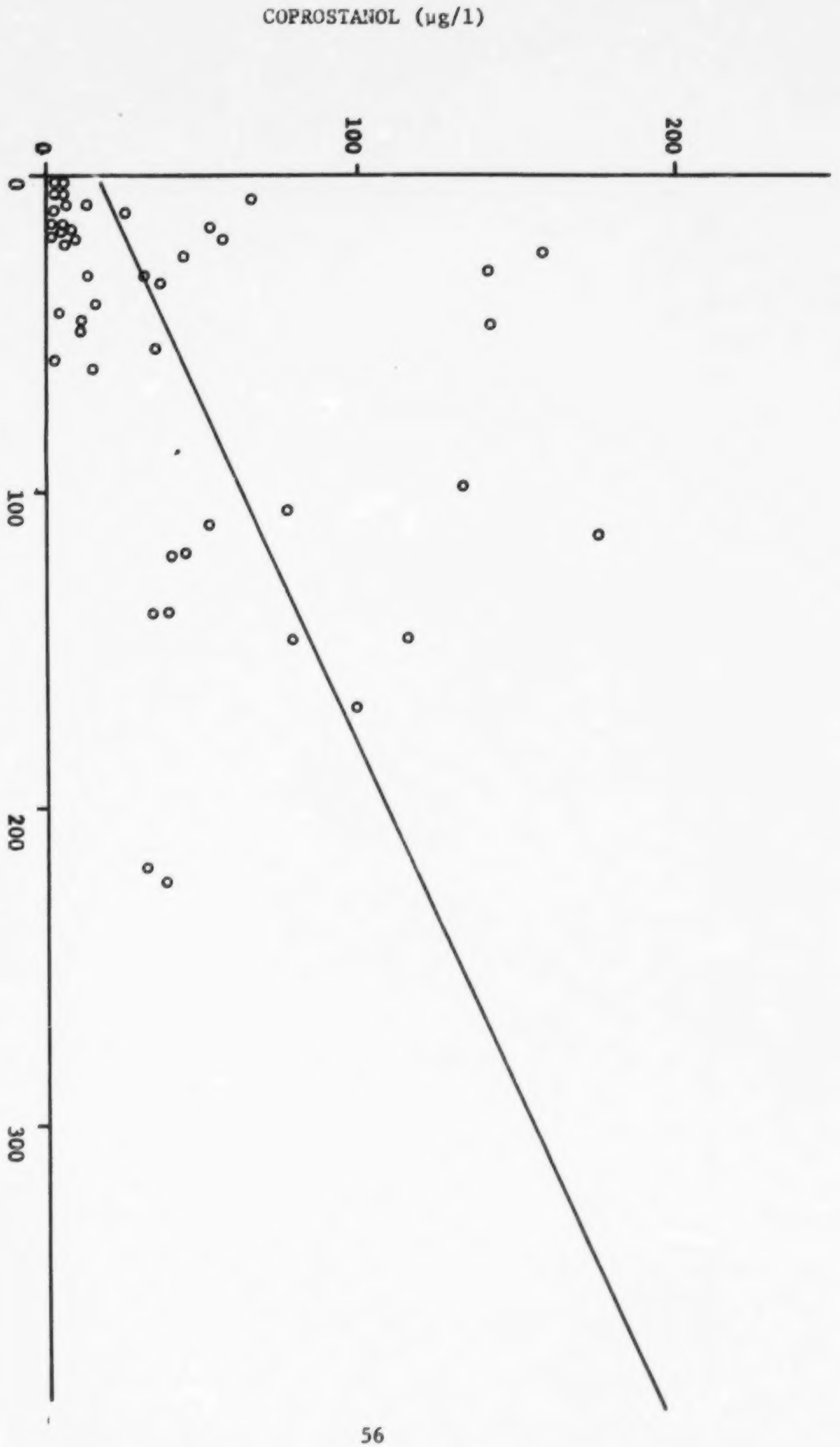


FIGURE 20. COPROSTANOL vs BOD OVERALL

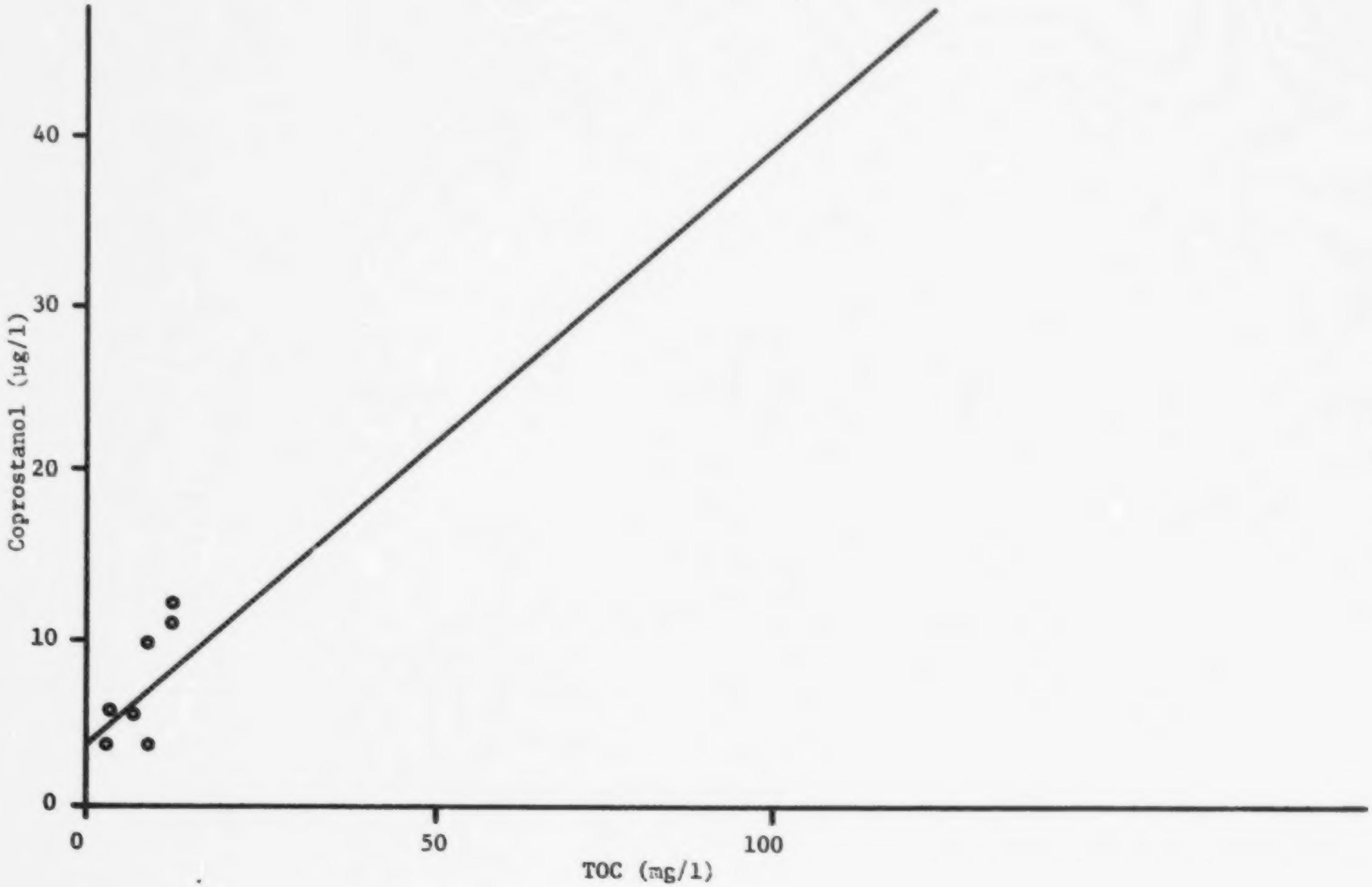


FIGURE 21. COPROSTANOL vs TOC SPECIAL STUDY ON MUNICIPAL CONTACT STABILIZATION PLANT

TABLE 20

SUMMARY OF STATISTICAL ANALYSES

Y: Coprostanol X_2: BOD
X_1: COD X_3: TOC

Experiment No.	Data Source	Coprostanol vs. COD	Coprostanol vs. BOD	Coprostanol vs. COD, BOD
1	Campus Primary Treatment	(1) $y = 23.5 - 0.197X + 0.00182X^2$ $R^2 = 0.878$, or (2) $y = -40.8 + 0.571X$ $R^2 = 0.851$ (Fig.)	(1) $y = 1.63 = 0.110X + 0.00245X^2$ $R^2 = 0.805$, or (2) $y = -0.313 + 0.668X$ $R^2 = 0.765$ (Fig.)	$Y = \beta_o + \beta_1 X_1 + \varepsilon$
2	Campus Contact Stabilization Plant	$y = -32.5 - 0.702X - 0.000666X$ $R^2 = 0.878$ (Fig.)	$y = 30.1 + 0.682X$ $R^2 = 0.369$ (Fig.)	$Y = \beta_o + \beta_1 X_1 + \beta_2 X_1{}^2 + \varepsilon$
3	Campus Trickling Filter Plant	$y = 33.0 - 0.183X + 0.00167X^2$ $R^2 = 0.706$ (Fig.)	$y = 16.8 + 0.432X$ $R^2 = 0.595$ (Fig.)	$y = -4.29 + 0.58X_1 - 1.77X^2$ $R^2 = 0.859$
4	Campus Secondary Treatment	$y = -0.186 + 0.625X - 0.000594X^2$ $R^2 = 0.719$ (Fig.)	$y = 26.9 + 0.461X$ $R^2 = 0.374$ (Fig.)	$y = 12.46 + 0.0959X_1 +$ $0.403X_2$ $R^2 = 0.470$
5	Campus Tertiary Treatment	(Fig.)	(Fig.)	$Y = \beta_o + \beta_1 X_1 + \beta_2 X_1{}^2$ $+ \beta_3 X_2 + \varepsilon$
6	Campus Plant Overall	$y = 0.1195 + 0.623X - 0.000587X^2$ $R^2 = 0.731$ (Fig.)	$y = 16.7 + 0.472X$ $R^2 = 0.709$ (Fig.)	$Y = \beta_o + \beta_1 X_1 + \varepsilon$

TABLE 20 (Cont.)

Experiment No.	Data Source	Coprostanol vs. COD	Coprostanol vs. BOD	Coprostanol vs. COD, BOD
7	Municipal Plant Overall	$y = 9.31 + 0.228X$ $R^2 = 0.640$ (Fig.)	$y = 0.950 + 0.383X$ $R^2 = 0.709$	$Y = \beta_o + \beta_2X_2 + \varepsilon$
8	Municipal Trickling Filter Plant	(Fig.)	$y = 18.0 + 0.841X$	$y = 11.6 - 0.694X_1$ $+ 1.10X_2$ $R^2 = 0.552$
9	Municipal Plant Overall	$y = 32.8 + 0.151X$ $R^2 = 0.311$	(1) $y = -3.19 + 1.24X - 0.00354X^2$ $R^2 = 0.527$ (2) $y = 19.3 + 0.421X$ $R^2 = 0.377$	**
10	Contact Stabilization Plant Overall	$y = -0.144 + 0.572X - 0.000550X^2$ $R^2 = 0.776$	$y = 24.1 - 0.404X$ $R^2 = 0.291$	$y = 8.23 + 0.101X_1$ $+ 0.333X_2$ $R^2 = 0.425$
11	Trickling Filter Plant Overall	$y = 7.89 + 0.437X - 0.000415X^2$ $R^2 = 0.405$	$y = 24.6 - 0.470X$ $R^2 = 0.459$	$Y = \beta_o + \beta_2X_2 + \varepsilon$
12	Secondary Treatment Overall	$y = -5.26 + 0.55X - 0.000528X$ $R^2 = 0.600$	$y = 24.4 + 0.438X$ $R^2 = 0.366$	$y = 15 + 0.087 + 0.357X_2$ $R^2 = 0.446$

**No relationships were found by the model given.

TABLE 20 (Cont.)

Experiment No.	Data Source	Coprostanol vs. COD	Coprostanol vs. BOD	Coprostanol vs. COD, BOD
13	Campus and Municipal Plants Combined	$y = -8.08 + 0.563X - 0.00535X^2$ $R^2 = 0.630$ (Fig.)	$y = 17.3 + 0.457X$ $R^2 = 0.429$ (Fig.)	$y = 8.85 + 0.100X_1 + 0.362X_2$
		Coprostanol vs. TOC	Coprostanol vs. TOC, COD, BOD	
14	Special Study On Municipal Contact Stabilization Plant	$y = 4.94 + 0.343X$ $R^2 = 0.977$ (Fig.)	$Y = \beta_o + \beta_3 X_3 + \epsilon$	

There is insufficient evidence to reject H_o at $\alpha = 0.05$.

Model II: $Y = \beta_o + \beta_1 X + \epsilon$

Prediction equation: $y = -40.8 + 0.571X$ (2)

$n = 8$ $R^2 = 0.851$

Test on dependency of y by two-sided student's t test:

H_o: $\beta_1 = 0$

$$\text{TS:} \quad t = \frac{\hat{\beta}_1 - 0}{\text{MSEC}_{11}} = 5.88$$

$t_{0.025,6} = 2.44$ H_o rejected.

Conclusion

Whether there is curvature or not will need more data points to verify.

Possibly, Coprostanol (Y) could be related to X(COD) by equation (2) as shown in Figure 17. Equation (1) was also plotted for reference.

b. Coprostanol vs. BOD

Y: Coprostanol
X: BOD

Model I: $Y = \beta_o + \beta_1 X^2 + \epsilon$

Prediction equation: $y = 1.63 - 0.110X + 0.00245X^2$ (3)

$n = 8$ $R^2 = 0.805$

H_o: $\beta_2 = 0$ Calculated $t = 2.55$

Critical t-values: $t_{0.025,5} = 2.57$, $t_{0.05,5} = 2.01$

There is insufficient evidenct to reject H_o at $X = 0.05$.

Model II: $Y = \beta_o + \beta_1 X + \epsilon$

Prediction equation: $y = -0.313 + 0.668X$ (4)

$n = 8$ $R^2 = 0.765$

H_o: $\beta_1 = 0$ Calculated $t = 4.45$

Critical $t_{0.025,6} = 2.44$ H_o rejected

Conclusion

Coprostanol could be related to BOD as shown in prediction (4) (Fig. 18). More data are needed to determine whether there is curvature or not.

c. Coprostanol vs. COD & BOD

Y: Coprostanol
X_1: COD
X_2: BOD

Complete model: $Y = \beta_o + \beta_1 X_1 + \beta_2 X_1{}^2 + \beta_3 X_2 + \beta_4 X_2{}^2 + \varepsilon$

Prediction equation: $y = 7.70 - 1.0X_1 + 0.00350X_1{}^2 + 1.31X_2 - 3.54X_2{}^2$ (5)

$n = 8$ $R^2 = 0.970$

Reduced model: $Y = \beta_o + \beta_1 X_1 + \beta_2 X_2 + \varepsilon$

Prediction equation: $y = -43.7 + 0.410X_1 + 0.225X_2$ (6)

$n = 8$ $R^2 = 0.871$

Analysis: Test on curvature (complete model): H_o: $\beta_2 = \beta_4 = 0$

TS: $F = \frac{\text{SS Drop}}{\text{SSE (complete model)}} = 3.36$

RR: $F > F_{2,1,2}$

Test on dependency (reduced model): H_o: $\beta_1 = 0$ $t = 6700$

H_o rejected Critical $t_{0.025,5} = 2.57$

H_o: $\beta_2 = 0$ $t = 0.900$ H_o accepted.

Conclusion

Coprostanol could be related to COD by $Y = \beta_o + \beta_1 X_1 + \varepsilon$.

Discussion of Results

1. Coprostanol vs. COD: The best fit was found in Experiment 1 (Campus Plant--Primary Treatment, $R^2 = 0.851$) and Experiment 2 (Campus Contact Stabilization Plant, $R^2 = 0.878$). Experiment 9 (Municipal plant, Overall, $R^2 = 0.311$) and Experiment 11 (Trickling Filter Plant, Overall, $R^2 = 0.405$) show lack of fit. No relationships were found in Experiment 5 (Campus Tertiary Treatment) and Experiment 8 (Municipal Trickling Filter Plant). Most of the models fitted show a parabolic relationship between coprostanol and COD.
2. Coprostanol vs. BOD: Good results were found only in Experiment 1 (Campus Plant--Primary Treatment, $R^2 = 0.765$) and Experiment 7 (Municipal Plant, Overall, $R^2 = 0.709$). No relationship was found in Experiment 5 (Campus Tertiary Treatment). Besides Experiment 5 and the uncertainty of Experiments 1 and 9, a straight line relationship was found to exist between coprostanol and BOD.
3. Coprostanol vs. COD and BOD: The best fit of the combined models was found in Experiment 3 (Campus Trickling Filter Plant, $R^2 = 0.595$). No relationship was shown in Experiment 9 (Municipal Plant, Overall).
4. Coprostanol vs. TOC: The coefficient of determination (R^2) in this analysis was found to be 97 percent which is a good fit. The model

showed that coprostanol and TOC might be correlated by straight line.
5. Coprostanol vs. TOC, COD and BOD: The results on the analysis of a combined model revealed that variation in the coprostanol value is accounted for mostly by TOC. COD and BOD contribute less information compared with TOC.

DEVELOPMENT OF A COLORIMETRIC METHOD

Summary of Results

A simple colorimetric method was devised for the analysis of coprostanol in sewage and polluted surface waters. The method eliminated interferences from cholesterol and other constituents by oxidation with CrO_3-pyridine and by simple chromatography on an alumina column. Although the reagent also oxidized coprostanol, the resultant coprostanone formed quantitatively and was separated by the alumina column.

The color was developed as follows: Coprostanone was reacted with 2,4-DNPH* to form the hydrazone. The hydrazone was hydrolyzed after excess 2,4-DNPH was removed. The liberated 2,4-DNPH was then oxidized with periodate to the diazonium salt and coupled with α-naphthylamine. The azo compound had intense absorption, with $\varepsilon = 35,000$, at 500 nm in an acidic solution of ethylacetate-acetic acid-concentrated HCl. The complete analysis required about 2 hours.

The method was applied to several field samples, with over-estimation of the coprostanol concentration by a factor of 1.6 as compared to the results obtained by GLC. Although some natural waters contained colorimetry-sensitive materials which were not coprostanol, the amount was insignificant. The compounds that interfere were shown to have a high probability of being of fecal or urinal origin.

Introduction

The gas-liquid-chromatographic (GLC) method for coprostanol analysis has major disadvantages for actual surveys of this indicator: GLC instrumentation is expensive and not always available to sewage treatment plants or field observers. The object of this part of the study was to develop a less expensive and simple, yet sensitive technique for the determination of coprostanol in sewage and surface waters.

Colorimetric techniques have been reported in the literature. Hanel and Dam [55] reported that coprostanol obtained from feces gave a "faint" reaction with a molar absorbence of 1066-930 at 528 nm the Tschugaeff reaction (with acetylchloride and zinc chloride in chloroform). Wells and Morres [56] evaluated the use of digitonide which forms insoluble complexes with sterols. They concluded that the technique was less applicable than GLC, because of the solubility of the coprostanyl digitonide and of the difficulties in removal of interfering cholesterol. They further reported that the detection limit was several hundred micrograms. This high level is found only in raw sewage or solid fecal material. The concentration of coprostanol in polluted water is usually much less than one hundred micrograms per liter. Gerson [57] applied the Zlatkis-Zak color development method to the analysis of coprostanol in fecal lipids. He stated that the detection limit was about 75 μg and that cholesterol interfered.

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*2,4-Dinitrophenylhydrazine

The principal difficulties in the colorimetric techniques for coprostanol analysis in sewage and surface waters are:
1. Removal of interfering materials, principally cholesterol, and
2. Discovery of a sensitive color reaction which enables the determination of less than one microgram of coprostanol.
To overcome the first problem, the oxidative decomposition of cholesterol followed by simple chromatographic removal of its oxidation products, together with the elimination of other interfering constituents were considered. Several oxidation methods were evaluated. To overcome the second problem a wide variety of color reactions were studied.

The following sections describe the optimization of the above steps, resulting in an analytical procedure that can quantitatively determine less than one microgram of coprostanol in sewage and surface waters.

Recovery of Coprostanol

Summary of Results

It was found necessary to saponify the samples because of high ratios of esterified to unesterified coprostanol in many samples. A simple saponification procedure was developed.

Procedure and Results

1. Evaluation of error in the extraction and washing steps. Because of its hydrocarbon nature, coprostanol is soluble in many organic solvents. Hexane has been used as the principal extraction solvent by most authors [8, 13, 58]. Hexane, however, can extract not only coprostanol but also many other organic constituents in waste waters and natural waters in which they are present in solution and as suspended solids. These organic materials interfere with the successful extraction of coprostanol by concentrating at the interface between the hexane and water layers. These materials also interfere with the spectrophotometric determination by generating color and turbidity. To eliminate these interfering materials, washing the hexane layer with 70 percent ethylalcohol [8] and with acetonitrile [59] has been used. The loss of coprostanol through the extraction-washing was evaluated. An experiment was conducted for the estimation of this error; the organic constituents in raw and treated sewage were

separated into four fractions by the procedure diagramed in Figure 22, i.e., hexane, 70 percent ethylalcohol, acetonitrile and residual water layers. The coprostanol concentrations were determined with GLC, after the extracts of each fraction were saponified, oxidized at 50°C, and the coprostanone fraction was separated, as discussed later. The results obtained are summarized in Table 21, and show that 5 to 23 percent of the coprostanol was lost by the above extraction and washing technique. Most of the error resulted from the solvent washing steps. The reduced recovery was unsatisfactory and could have been caused by the solubility of free coprostanol in the washing solvents; however, the error was too high. A reasonable explanation for this error is based on the observation of higher levels of insoluble materials at the interface between the two solvent layers in the case of samples that had not been diluted. This suggests that coprostanol esters present in the water, are so soluble in more polar solvents that they would be included in the solids at the interface. This would result in the high errors observed. In the case where the concentration of coprostanol is low, for example, 6 μg/l in the sample after the oxidation pond, the low recovery seems to be attributable both to extraction of free coprostanol with the solvents and to the high ratio of esterified coprostanol to free coprostanol. The amount of free coprostanol, expected to be extracted by the solvents was calculated as 0.7 μg/l, based on the assumption that the equilibrium ratio in hexane and the washing solvents was 91.5 and 8.5 percent respectively, by the following calculation:

$$(6.0 + 1.8)\ \mu g/l \times \left(\frac{8.5\%}{91.5\%}\right) = 0.72\ \mu g/l$$

Although this estimation is very rough, it supports the suggested interpretation, i.e., the existence of esterified forms of coprostanol.

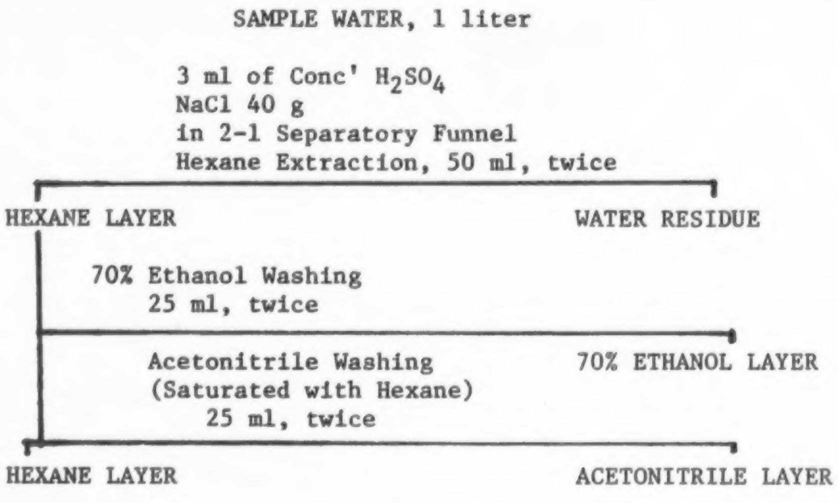

SAMPLE WATER, 1 liter
3 ml of Conc' H_2SO_4
NaCl 40 g
in 2-1 Separatory Funnel
Hexane Extraction, 50 ml, twice
HEXANE LAYER
WATER RESIDUE
70% Ethanol Washing
25 ml, twice
Acetonitrile Washing
(Saturated with Hexane)
25 ml, twice
70% ETHANOL LAYER
HEXANE LAYER
ACETONITRILE LAYER

FIGURE 22. SCHEME FOR THE FRACTIONATION

TABLE 21

ESTIMATION OF ERROR IN RECOVERY BY SOLVENT EXTRACTION AND WASHING[a]

	Recovery of Coprostanol							
Layers	Raw Sewage I[b]		Raw Sewage II[c]		Effluent		After Oxidation Pond	
	µg/l	%	µg/l	%	µg/l	%	µg/l	%
Hexane	371.0	86	17.1	95	15.1	91	6.0	77
70% Ethanol	39.7	9.2	<1.0[d]		<1.0[d]		<1.0[d]	
Acetonitrile	19.5	4.5	<1.0		<1.0		<1.0	
Residual Water	1.8	0.4	<1.0		<1.0		<1.0	
Total loss	61.0	14	0.9	5	1.5	9	1.8	23

a. The sampling stations were at the University of Florida, Campus Treatment Plant, Gainesville, Florida.

b. One liter was extracted without dilution (week day samples).

c. 200 ml of raw sewage was diluted to one liter (weekend samples).

d. Less than one microgram of coprostanol could not be determined by GLC with FID; the coprostanol content was measured by combining three samples from 70 percent ethanol, acetonitrile and residual water.

2. Effect of Saponification. As was discussed in the previous section, coprostanol occurred in sewage and surface waters, principally in the free state. The remainder is present in more complex forms, probably as esters. Rosenfeld [15] estimated that 30 percent of the total coprostanol in fecal sterols is in the form of esters. One method used for saponification was refluxing for 3 hours with 7.5 percent potassium hydroxide in 70 percent ethanol [5]. This mild saponification technique seems to be satisfactory for the recovery of esterified coprostanol, however, it takes too much time. A more rapid saponification method has been found which will saponify the esterified coprostanol within a few minutes. The procedure is simple, i.e., the well-dried hexane extracts in a test tube were heated to boiling on a flame or in a water bath after 1 ml of 0.5N sodium hydroxide in absolute methanol had been added. The volume was reduced to about 0.2 ml. This method was used to determine the amount of esterified coprostanol in field samples. The results are shown in Table 22 for samples that were not preserved with H_2SO_4.

The saponifiable coprostanol in raw sewage and treatment plant effluent, in the case of unpreserved samples (Table 22), were 25 to 30 percent. These values are the same as reported to Rosenfeld [15], however the samples which were obtained from the receiving stream or the samples which were acidified with concentrated H_2SO_4 (Table 23), showed higher ratios, 45 to 55 percent. These higher ratios could have been due to esterification of the free coprostanol by the action of the acid on other constituents in the sewage and natural waters or to the formation of sulfate esters. The high ratio present in the stream sample might have been due to the chlorination process in the treatment plant, or to some unknown stream conditions such as bacterial actions or chemical reactions between free coprostanol and other components of natural waters.

Because the acidification of sample water is a necessary step for long-term preservation to prevent bacterial decomposition of coprostanol, it is obvious that the saponification procedure is required in the analysis, if exact quantitative results are desired.

Oxidation of Cholesterol and Coprostanol

Summary of Results

Cholesterol was separated from coprostanol by oxidation with the Sarett reagent (analydrous chromic acid--pyridine complex in glacial acetic acid) followed by adsorption of the oxidation product of cholesterol on an alumina column.

Procedures and Results

The removal of cholesterol was one of the major objectives of this part of the study. It is difficult to separate coprostanol and

TABLE 22

EFFECT OF SAPONIFICATION

Sample[a]	Saponified (μg/1)	Unsaponified (μg/1)	Saponifiable Coprostanol (%)
Raw Sewage[b]	368	266	27
Treatment Plant Effluent[b]	78	59	24
Receiving Stream[c]	117	66	43

[a] The samples were analyzed within 30 minutes after sampling, without adding 3 ml concentrated H_2SO_4.

[b] The City Sewage Treatment Plant, Gainesville, Florida.

[c] Sweet Water Branch, 1/4 mile down stream from the Gainesville Sewage Treatment Plant outfall.

When the samples were preserved by adding 3 ml of concentrated H_2SO_4, the amounts of the saponifiable coprostanol were about one half of the total coprostanol, as shown in Table 23.

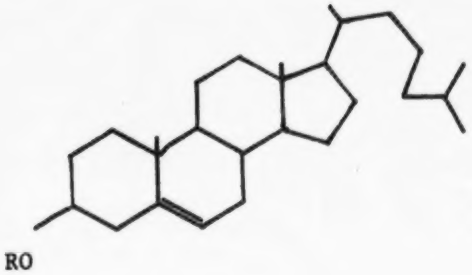
TABLE 23

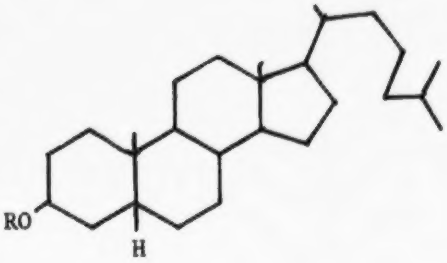
EFFECT OF SAPONIFICATION ON PRESERVED SAMPLES*

Sample	Saponified (μg/1)	Recovery of Coprostanol Unsaponified (μg/1)	Saponifiable Coprostanol (%)
Raw Sewage	490	247	49.7
Raw Sewage	478	212	55.6
Mean	484	230	52.4

*The samples were acidified with 3 ml of concentrated H_2SO_4, and analyzed after several hours. The samples were obtained at the City Sewage Treatment Plant, Gainesville, Florida.

cholesterol by liquid column chromatography because of their similar reaction with the columns; however, cholesterol (I) has a double bond at Δ^5, which will react differently with certain reagents than will coprostanol. It can be inferred that there should be an appropriate agent which is selective to cholesterol.


I. R = H: Cholesterol
R = OAc: Cholesterylacetate


II. R = H: Coprostanol
R = OAc: Coprostanylacetate

For example, ozone, osmate or permanganate will react only with the double bond of cholesterol. Preliminary experiments showed that cholesterol was oxidized by the modified Remieu-von Rudloff reagent (periodate with a catalytic amount of permanganate), [60, 61, 62] and coprostanol (II) was unchanged when the alcoholic groups of the sterols were protected by acetylation. Chromic acid anhydride oxidation in glacial acetic acid also showed the same results on the acetates of these sterols. These reagents were not useful for analysis, because coprostanylacetate (II) has a low color intensity. The molar extinction coefficient was determined to be 1,150 when color development by the Zlatkis-Zak method was used. This was lower than that of coprostanol itself.

On the other hand, both cholesterol and coprostanol have alcoholic hydroxy group at C_3 as another reactive site. Fieser [63] reported that the initial oxidation product of cholesterol with chromic acid is the ketone, Δ^5-Cholesten-3-one (III), and Ellis and Petrow [64] reported that they obtained the 3-keto-cholesterolepoxide from "α"-epoxy-cholesterol by milder oxidation with the chromic oxide-pyridine complex. This evidence indicates that the alcoholic groups of the sterols can be converted to the ketones by mild oxidation. The Sarett reagent, anhydrous chromic acid-pyridine complex in glacial acetic acid, was studied with the following results; cholesterol was oxidized rapidly along with the rapid conversion of coprostanol to coprostanone (IV). In Figure 23 the recoveries of cholesterol, coprostanol and coprostanone are plotted with reaction time.

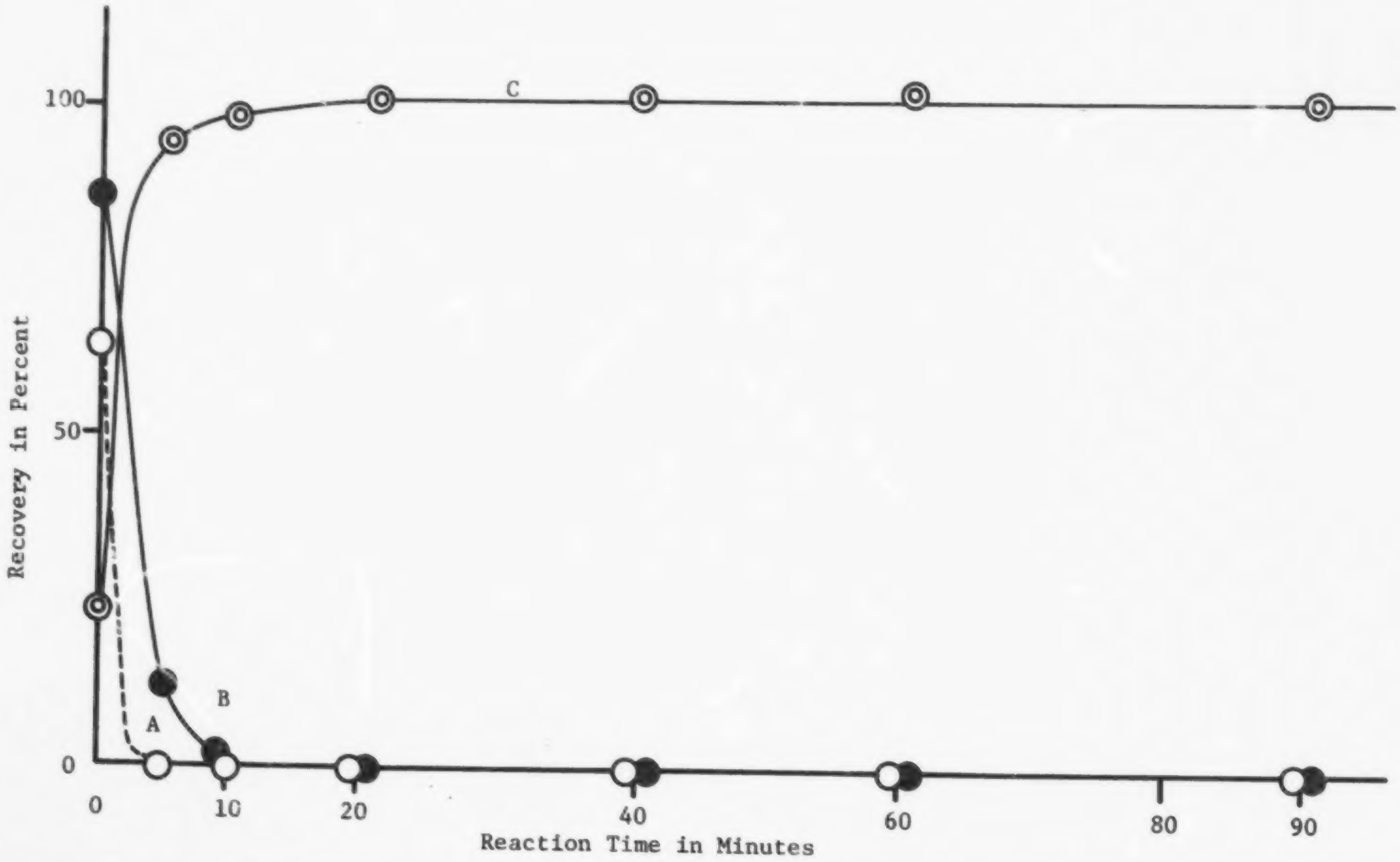
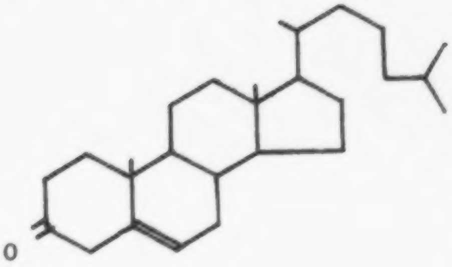
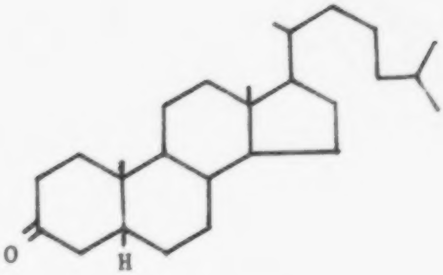


FIGURE 23. OXIDATION OF STEROLS AND FORMANTION OF COPROSTANONE WITH THE SARETT REAGENT AT 20°C

A: Coprostanol B: Cholesterol C: Coprostanone

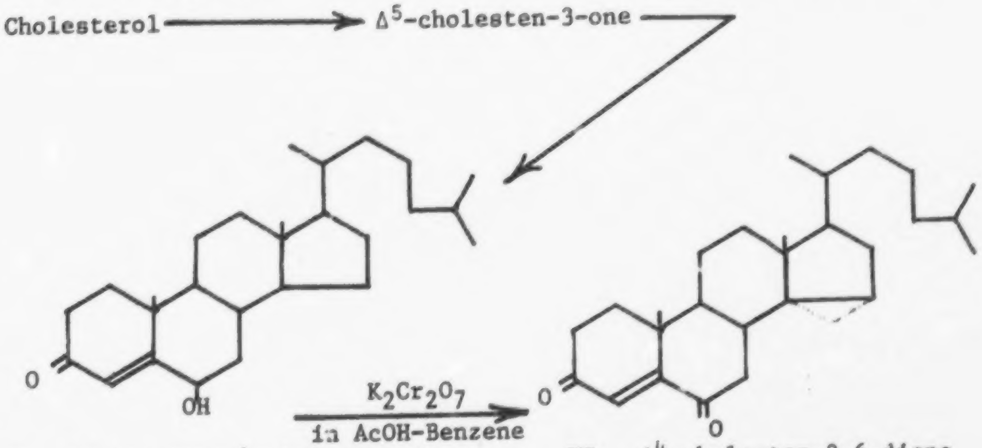


III. Δ^5-Cholesten-3-one



IV. Coprostanone

While the formation of coprostanone was quantitative and coprostanone itself was resistant to the oxidation reagent, the formation of Δ^5-cholesten-3-one was not quantitative. At least two oxidation products of cholesterol in the hexane extract were identified gas chromatographically as shown in Figure 24. One of these has been identified as Δ^5-cholesten-3-one (III), by comparing with the retention time of the authentic compound on the gas-chromatograph. This compound appeared dominantly at the early stage of the oxidation, i.e., in a few minutes. This identification is supported by Fieser [63] as he reported that the initial product of the oxidation of cholesterol was Δ^5-cholesten-3-one, which undergoes further oxidation to 6β-hydroxy-Δ^4 cholesten-3-one (V) and then to Δ^4-cholesten-3,6-dione (VI):



V. 6β-hydroxy-Δ^4-cholesten-3-one VI. Δ^4-cholesten-3,6-dione

The other oxidation product may have been the second product, 6β-hydroxy-Δ^4-cholesten-3-one (V). This conclusion is supported by Ellis and Petrow [64], who could isolate only 6β-hydroxy-Δ^4-cholesten-3-one in

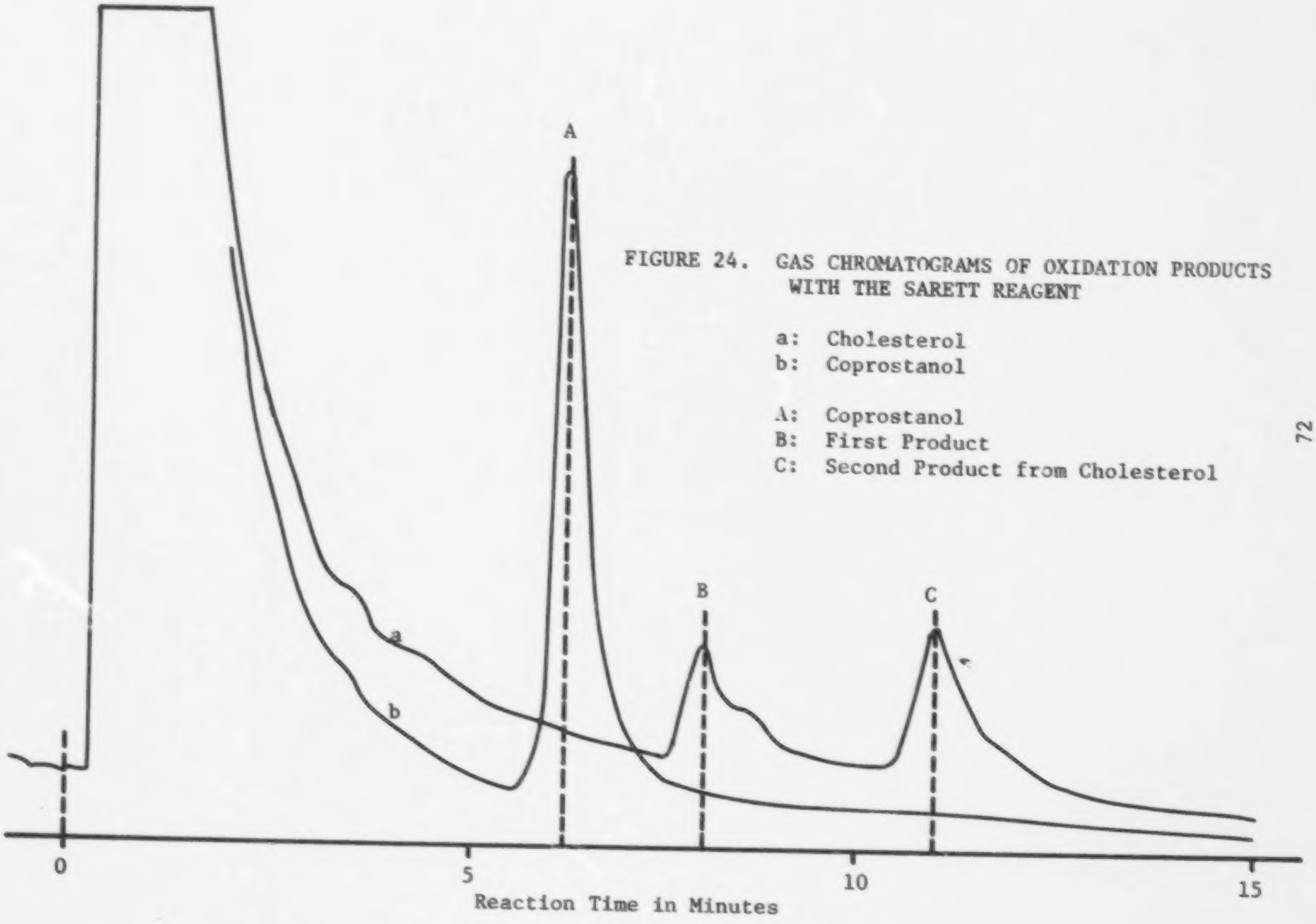


FIGURE 24. GAS CHROMATOGRAMS OF OXIDATION PRODUCTS WITH THE SARETT REAGENT

a: Cholesterol
b: Coprostanol

A: Coprostanol
B: First Product
C: Second Product from Cholesterol

the oxidation of "β"-epoxy-cholestan-3-ol with the chromic acid-pyridine complex. Because the oxidation with chromic acid-pyridine complex in glacial acetic acid is mild, it does not cause further oxidation of 6β-hydroxy group to the ketone. In Figure 25, the changes in these two products are plotted with reaction time. After cholesterol disappeared, the amounts of these two products increased, with the rate of formation of Δ^5-cholesten-3-one greater than that of 6β-hydroxy-Δ^4-cholesten-3-one.

These oxidation products can form hydrazones with 2,4-dinitrophenyl hydrazine (DNPH) as orange precipitates, and interfere with the colorimetric determination of coprostanol. The hydrazone of Δ^5-cholesten-3-one is less soluble in hexane than that of coprostanone, but it can be partially extracted with the solvent when the hydrazone of coprostanol is separated from unreacted 2,4-DNPH reagent. The problem then becomes how to remove these interfering oxidation products.

It was discovered that the second oxidation produce, 6β-hydroxy-Δ^4-cholesten-3-one, could be eliminated by passage through an alumina column, i.e., the product was eluted into the free sterol fraction with pure ethylacetate as shown in Table 24. On the other hand, the first product and also pure Δ^5-cholesten-3-one, behaved on the column similar to coprostanone, and could not be eliminated by the alumina column.

The product, Δ^5-cholesten-3-one, was more reactive toward oxidation reagents and should undergo further oxidation to the second product. As is seen in Figure 25, the first oxidation product reached the maximum yield in 20 minutes of reaction time and was present even after 40 minutes at the reaction temperature of 20°C. From this evidence, it could be expected that the yield of the first product would become negligible, if the reaction proceeded longer or if the temperature were increased. As longer reaction times than 30 minutes are not suitable for practical analysis, the effects of temperature on the fate of the first product, Δ^5-cholesten-3-one at 40°, 60° and 80°C was studied.

The changes of the oxidation products of cholesterol at various temperatures are plotted against reaction time in Figure 26 and 27. The first product, Δ^5-cholesten-3-one, in Figure 26, decomposed completely within ten minutes, when the temperature was over 60°C. However, at 40°C, it was present for over twenty-five minutes with the maximum yield at ten minutes. When the temperature was high, 80°C, the first product was oxidized within five minutes. Figure 27 indicates the change in yield of the second product with reaction time at various temperatures.

The above experiment indicated that cholesterol can be eliminated by oxidation with Sarett reagent at a temperature of 60°C with at least ten minutes reaction time. However, coprostanol, when oxidized at 80°C, decomposed and the recovery of coprostanone decreased to 85 percent

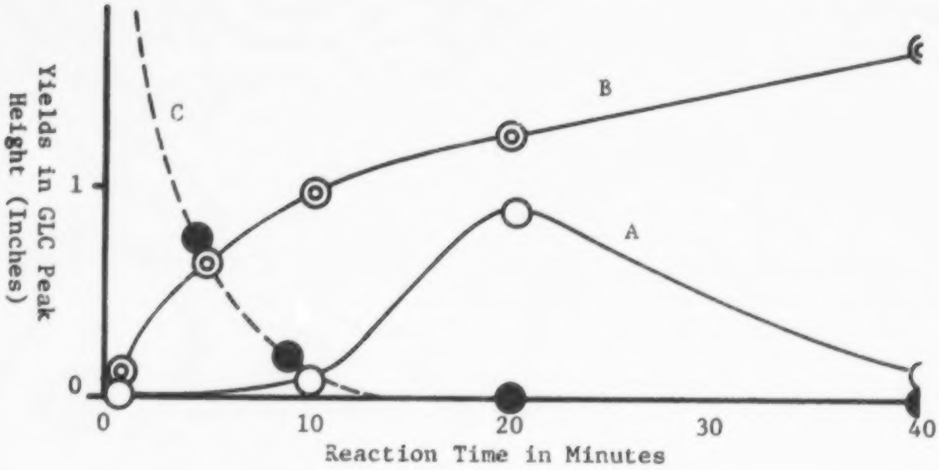


FIGURE 25. YIELDS OF THE OXIDATION PRODUCTS OF CHOLESTEROL WITH THE SARETT REAGENT AT 20°C

A: The First Product B: The Second Product C: Cholesterol

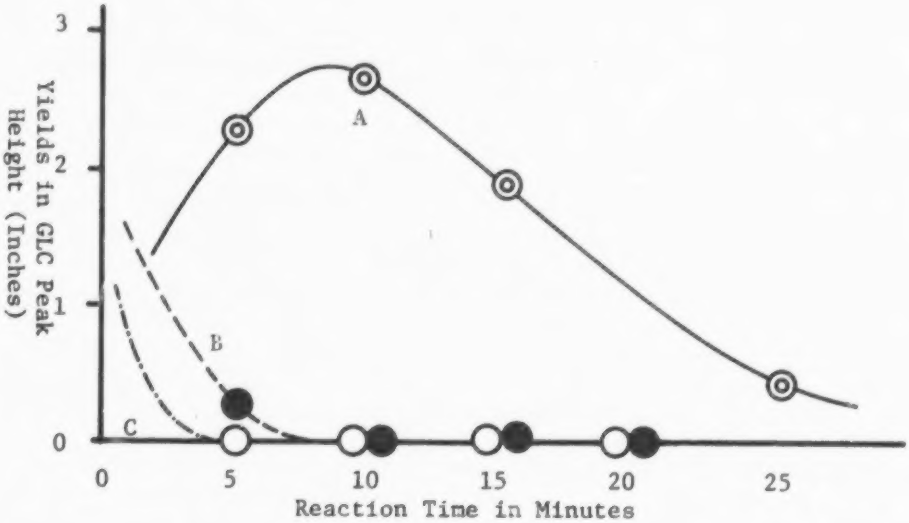


FIGURE 26. YIELDS OF THE FIRST OXIDATION PRODUCT OF CHOLESTEROL AT VARIOUS TEMPERATURES

A: 40°C B: 60°C C: 80°C

TABLE 24

Elution Pattern on an Alumina Column**

Solvent	Elution Volume (ml)	Cholesterol and Coprostanol	Coprostanone	The First Product and Δ^5-cholesten-3-one	The Second Product	Hydrocarbons (C_{20} and C_{28})
Hexane	5.0	-*	-	-	-	-
	5.0	-	-	-	-	-
5% Ethyl-acetate	2.0	-	+	+	-	-
	2.0	-	±	±	-	-
	2.0	-	-	-	-	-
	2.0	-	-	-	-	-
	2.0	-	-	-	-	-
Ethylacetate	2.0	+	-	-	+	-
	3.0	-	-	-	-	-

* + Positive; ± Trace; - Negative.

** The moisture content of alumina absorbent was critical. When the alumina was moistened in the atmosphere overnight, the separation of coprostanone failed, and 5 percent ethylacetate eluted the sterols and the second oxidation product of cholesterol. The absorbent must be used fresh or the moisture content controlled by the procedure in Appendix-6.

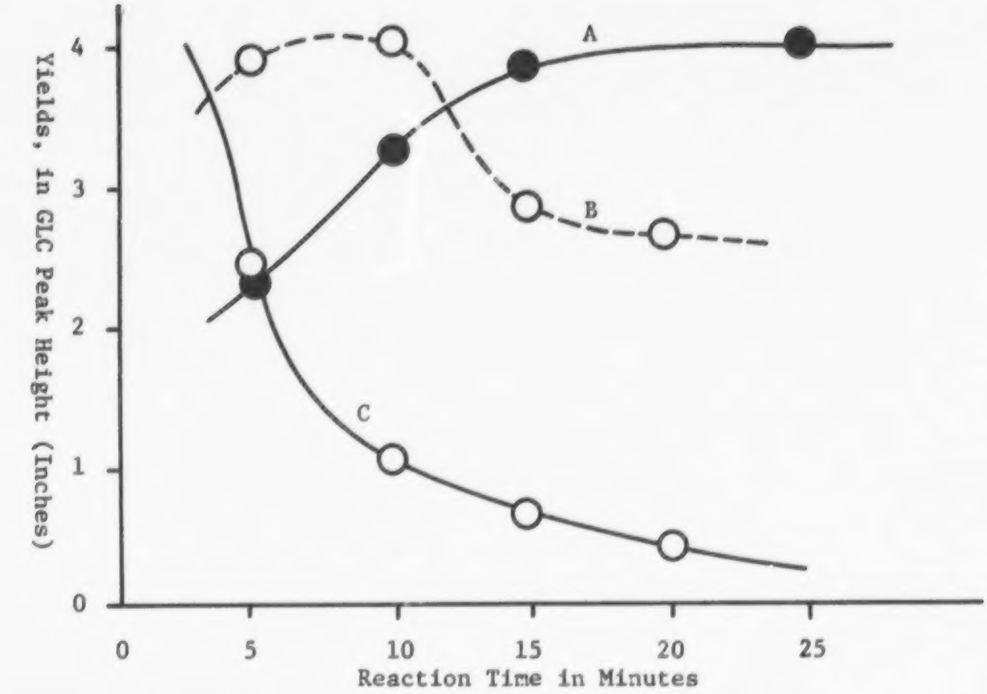


FIGURE 27. YIELDS OF THE SECOND OXIDATION PRODUCT OF CHOLESTEROL AT VARIOUS TEMPERATURES
A: 40°C B: 60°C C: 80°C

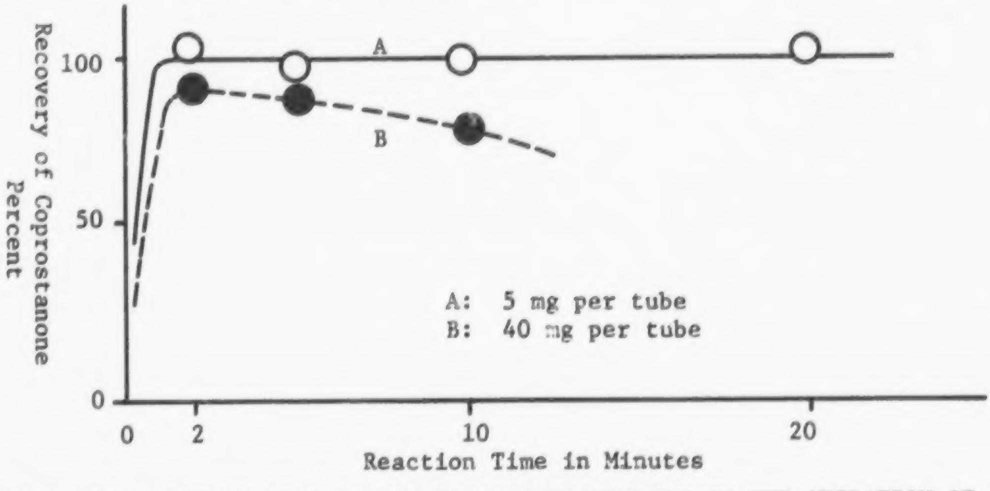
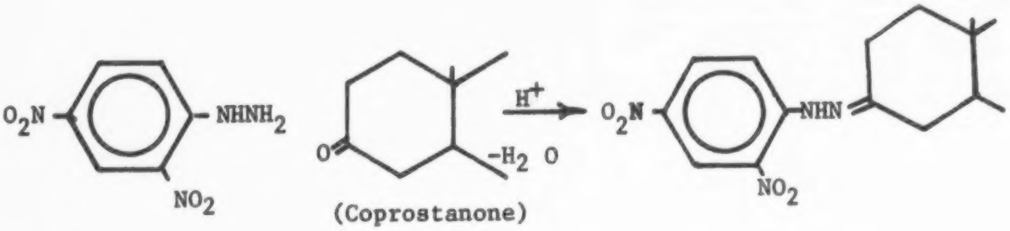


FIGURE 28. EFFECT OF C_rO_3 DOSAGE ON COPROSTANONE IN THE OXIDATION OF STEROLS WITH THE SARETT REAGENT AT 80°C

after ten minutes as shown in Figure 28. From this observation, it was found that the reaction temperature was critical and must be optimized so as to decompose the first product but not to degrade the resultant oxidation product of coprostanol, coprostanone.

Formation of Coprostanone-2,4-Dinitrophenylhydrazone and Removal of Unreacted 2,4-DNPH

2,4-Dinitrophenylhydrazine (2,4-DNPH) (VII), which is widely used for the characterization of carbonyl compounds, was employed in this study in absolute methanol solution*[64,65]. The coprostanone-2,4-DNPH' (VIII), formed as shown below, is a yellow precipitate that is sparingly soluble in water, but very soluble in many organic solvents such as hexane, acetic acid and acetonitrile, even though it has polar substituents in the phenylhydrazine moiety.



VII. 2,4-DNPH VIII. Coprostanone-2,4-DNPH'

The formation of coprostanone-2,4-DNPH' seemed to be quantitative, since the hexane extract from the reaction solution did not show the presence of coprostanone.

2,4-DNPH is soluble in acidic solution, however, hexane partially extracts the reagent, and may cause a high reagent background in the measurement of the developed color. Techniques for removal of the reagent have been proposed in the literature by absorption chromatography [66,67] and oxidative decomposition [68] of the reagent. According to Reich, et. al., [69] 2,4-DNPH can be removed by oxidation with either Fehling's solution or Benedict's reagent, producing m-dinitrobenzene. A new approach was tried in this study, i.e., oxidative decomposition of the unreacted 2,4-DNPH. Several oxidation reagents were tried. Preliminary experiments showed that both potassium dichromate in acidic solution and hydrogen peroxide in alkaline solution gave satisfactory results. The conditions required to obtain a reasonably low reagent background were determined for dichromate oxidation.

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* 95 percent ethanol is commonly used as a hydrazonation solvent, but this solvent gives problems with the oxidative removal of unreacted 2,4-DNPH reagent with acidic dichromate. The 2,4-DNPH does not dissolve completely in 2N H_2SO_4 solution, in which the coprostanone-2,4-DNPH is suspended, and results in incomplete decomposition of unreacted 2,4-DNPH reagent.

In Table 25, the effects of extraction conditions are summarized. When the unreacted 2,4-DNPH reagent was not oxidized with acidic dichromate, the reagent blank was high, E_{rb}^{10} = 0.570, however, when it was oxidized the blanks were greatly reduced. In the case of extraction from alkaline solution reagent blanks of zero were obtained.

The effect of temperature on the removal of unreacted 2,4-DNPH reagent is shown in Table 26. It shows measurable values for the reagent blanks at temperatures below 60°C; however, when the temperature was 80°C, the reagent blank became zero.

TABLE 25

EFFECT OF EXTRACTION CONDITIONS FOR COPROSTANONE-2,4-DNPH

Dilution Solvent	Reagent Blank Value* E_{500}nm vs. Distilled Water
Not oxidized with Dichromate:**	0.570
Distilled Water	0.570
Oxidized with Dichromate:	
2N H_2SO_4	0.032
12N H_2SO_4	0.010
4N NaOH	0.000
Solvent***	0.000

* The absorbence in 10 ml of solution. The color development procedure followed was the method discussed in the next section.

** After the hydrazonation, the solvent was diluted with 10 ml of carbonyl-free water without adding dichromate solution or 5 ml 2N H_2SO_4 solution.

*** Ethylacetate-acetic acid concentrated HCl (6:3:1, by bolume).

TABLE 26

TIME AND TEMPERATURE EFFECTS ON REAGENT BLANKS

Times in Minutes	Reagent Blanks		(E_{500}nm/10 ml)	
	20°C	40°C	60°C	80°C
5	0.017	0.010	0.007	0.000
10	0.016	0.007	0.008	-----
20	0.014	0.009	0.006	-----

* The color development method was the same as in note (*) of Table 25.

Color Development

1. The Color of Coprostanone-2,4-Dinitrophenylhydrazone in Basic Solvent Systems--Coprostanone-2,4-DNPH is soluble in hexane and shows a yellow color, which has no absorption maximum in the visible region but does have a small shoulder near the ultra violet region, at 415-390 nm, with a molar absorbence of 7,270. As this shoulder is too close to the UV region and the absorption intensity is too low, it was difficult to use this band for analysis.

The Janovsky reaction [70] of m-dinitro compounds was considered, in which an alkali-acetone solution (5 ml of acetone and 2 ml of 5 percent sodium hydroxide) was employed. The resultant wine-red color was unstable and started to fade in a few minutes. In order to find a suitable solvent to stabilize the color of coprostanone-2,4-DNPH, alkaline solutions of acetonitrile and pyridine were prepared and evaluated. The brown to violet colors were measured on a spectrophotometer at 465 nm in a 1 cm cell.

The results are summarized in Table 27 and indicate that the acetonitrile system was not suitable because of a low absorption intensity, $E^{10}_{\mu g} = 0.001$.* The pyridine system resulted in a high absorption intensity, $E^{10}_{\mu g} = 0.014$, but had a high reagent background, $E^{10}_{rb} = 0.112$.** This high reagent background was considered to be due to m-dinitrobenzene which was formed from unreacted 2,4-DNPH reagent by oxidation. In spite of the high absorption intensity of the colored product this technique cannot be used for the determination of amounts of coprostanol of one microgram or less because of the high reagent background.

2. Acid Hydrolysis of Coprostanol-2,4-DNPH', and Formation of Azo Compounds--Arylhydrazines have been known to be oxidized easily with many oxidizing agents such as selenious acid and arsenic acid, [71] forming the corresponding diazonium salts, which undergo coupling with aromatic amines and phenols forming azo compounds. The azo compounds have intense colors leading to their extensive use in analysis. For example, nitrites in the sea water [72], and nitrogen oxides in the atmosphere [73] are determined by use of this class of reaction. Feigl [71] states that arylhydrazones are detectable by coupling with α-naphthylamine after they are saponified with concentrated hydrochloric acid and the liberated hydrazines are oxidized with selenious acid to the diazonium salts. The hydrolysis of coprostanone-2,4-DNPH' with concentrated hydrochloric acid was shown to result in poor recovery of the hydrazine due

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* $E^{10}_{\mu g}$ is defined for convenience, as the absorbence of one microgram (coprostanol equivalent) of color materials in 10 ml of the solution, with 1 cm light path.

** E^{10}_{rb} indicates the absorbence of blanks in 10 ml solution at the maximum absorption wave length of the color materials, with 1 cm light path, in this case at 465 mμ.

TABLE 27

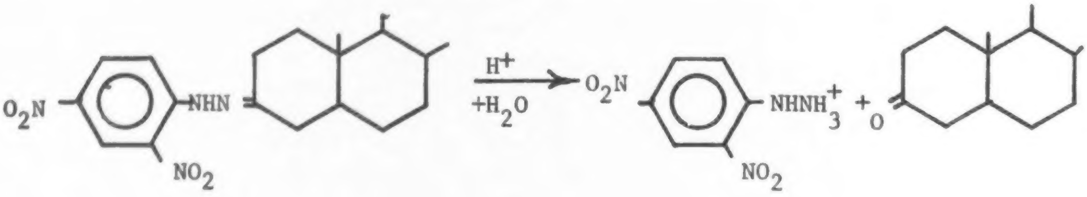
ABSORPTION INTENSITIES AND REAGNET BLANKS OF COLOR DEVELOPMENT TECHNIQUES IN BASIC SOLVENT SYSTEMS

Solvent System*		Maximum Wave Length, nm	Absorption ($E°\lambda max$)**	Intensity ($E^{10}_{\mu g}$)	Reagent Blank (E^{10}_{rb})
A. Acetonitrile	4.0 ml				
Methanol 0.5N	1.0 ml				
KOH	1.0 ml	465	3,680	0.001	0.050
B. Pyridine	3.0 ml				
1N KOH in MeOH	1.0 ml				
95% Ethanol	2.0 ml	465	53,000	0.014	0.112

* The solvent systems were designed to maintain alkaline conditions and to dissolve the resultant m-dinitrobenzene from unreacted 2,4-DNPH reagent.

** Molar extinction coefficient.

to the stability of the hydrazone and the reduced solubility in concentrated hydrochloric acid. The maximum recoveries were 90 percent.



Coprostanone-2,4-DNPH' 2,4-DNPH Coprostanone

Several solvents which might dissolve the hydrazone, were studied. Equal volumes of these solvents were mixed with concentrated hydrochloric acid. The solvents were: acetonitrile, methanol, ethanol, butanol, octanol, tetrahydrofurane, chloroform, acetic acid and propionic acid. The best solvent system was a mixture of acetic acid and concentrated hydrochloric acid (1:1) which led to quantitative hydrolysis as shown in Figure 29. Experiments were conducted to optimize the conditions, i.e., time and temperature, in the hydrolysis of coprostanone-2,4-DNPH with this solvent system. The results are shown in Figure 30.

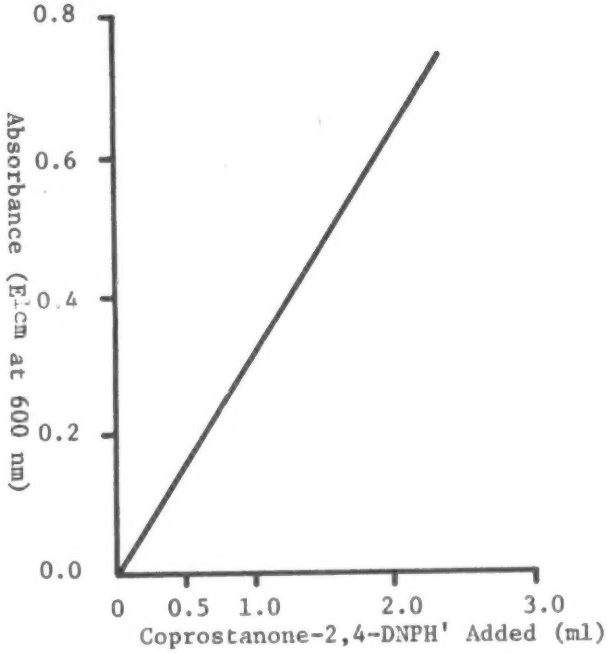


FIGURE 29. HYDROLYSIS OF COPROSTANONE-2,4-DNPH' WITH ACETIC ACID -- CONCENTRATED HCl (1:1)

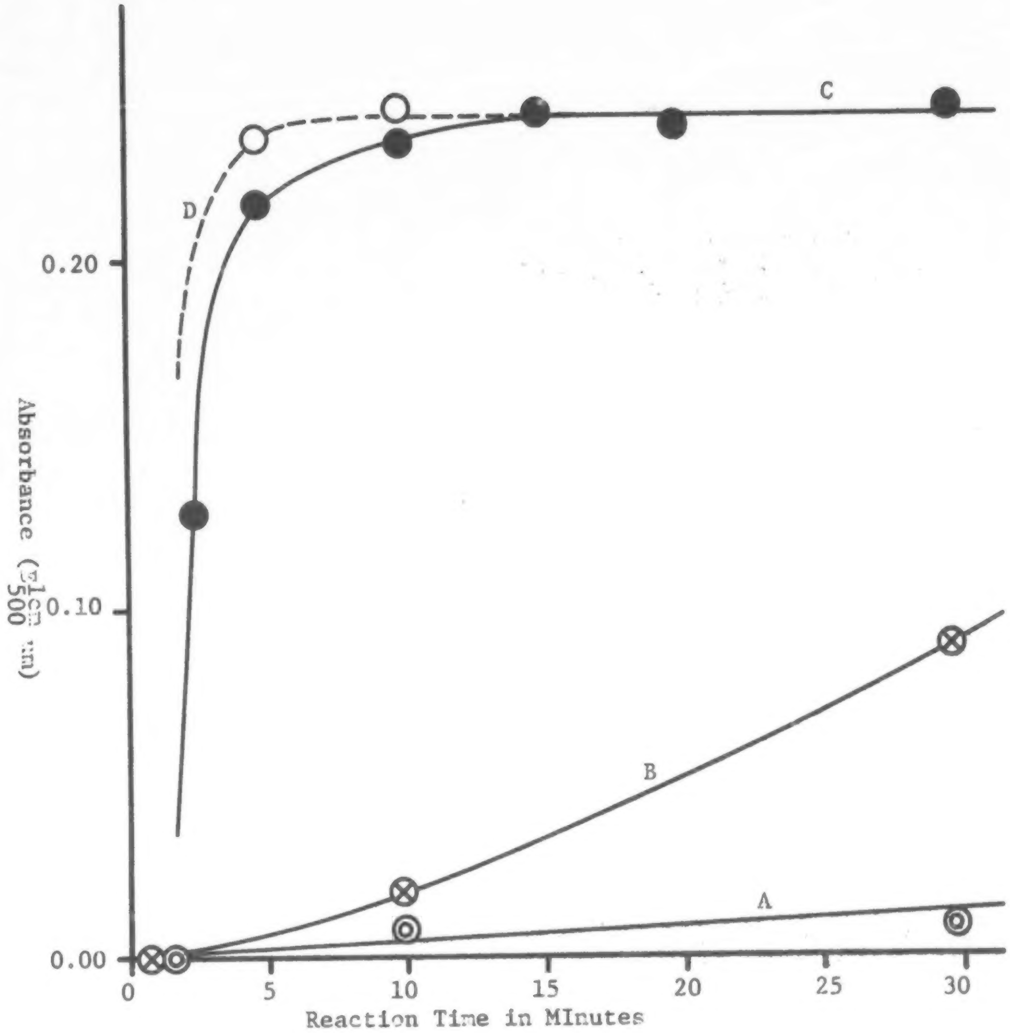
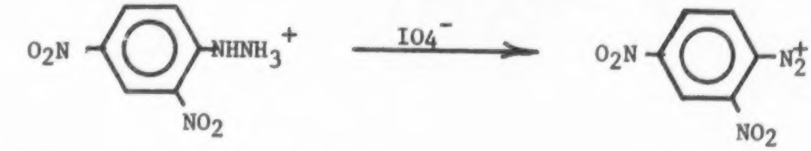


FIGURE 30. EFFECT OF TEMPERATURE AND TIME ON THE HYDROLYSIS OF COPROSTANONE-2,4-DNPH'

A: 20°C B: 50°C C: 90°C D:100°C

When the temperature was low, 20° and 50°C, the hydrolysis proceeded slowly and was not complete even after 90 minutes; however, at higher temperatures, 90° to 100°C, the hydrolysis was completed within 15 minutes. From this study the optimum hydrolysis conditions can be specified as: 100°C (boiling water) for ten minutes with 1:1 HCl-glacial acetic acid.

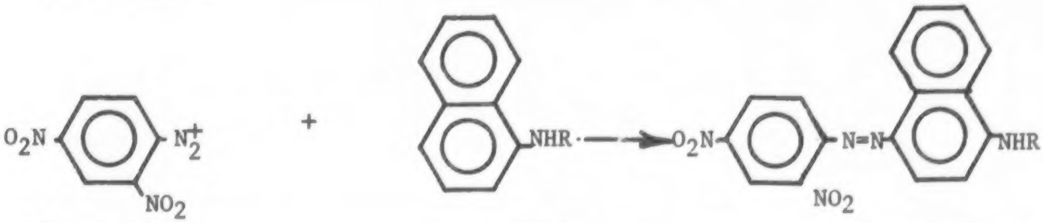
As the oxidation reagent to convert hydrazine to the diazonium salt, Feigl [71] recommended selenious oxide, a weak oxidizing agent. Since selenious oxide was not available, sodium periodate solution was used. The periodate oxidized 2,4-DNPH to the diazonium salt almost instantaneously and quantitatively, as shown in Figure 29.



2,4-DNPH-hydrochloride

Diazonium salt

Both α-naphthylamine and 1-(N-naphthyl)-ethylenediamine were evaluated as coupling agents for the analysis, by comparing their color intensities and reagent backgrounds. The absorption spectra are shown in Figures 31 and 32.



Diazonium Salt

R=H: α-naphthylamine
R=$CH_2CH_2NH_2$:

1-(N-naphthyl)-ethylene diamine

Azo-Compounds

In Table 28, the results are summarized, which indicate that the measurement of the color of the azo compounds of α-naphthylamine in acidic solution was best, because of the high absorption intensity and low reagent blank. With this color development method, it is possible to determine one microgram of coprostanol; $E^3_{\mu g}$ = 0.046 with reagent blank E^3_{rb} - 0.004. The reagent blanks in basic solution gradually increased in contact with the atmosphere. This phenomenon seems to have been caused by the oxidation of the naphthylamines by oxygen.

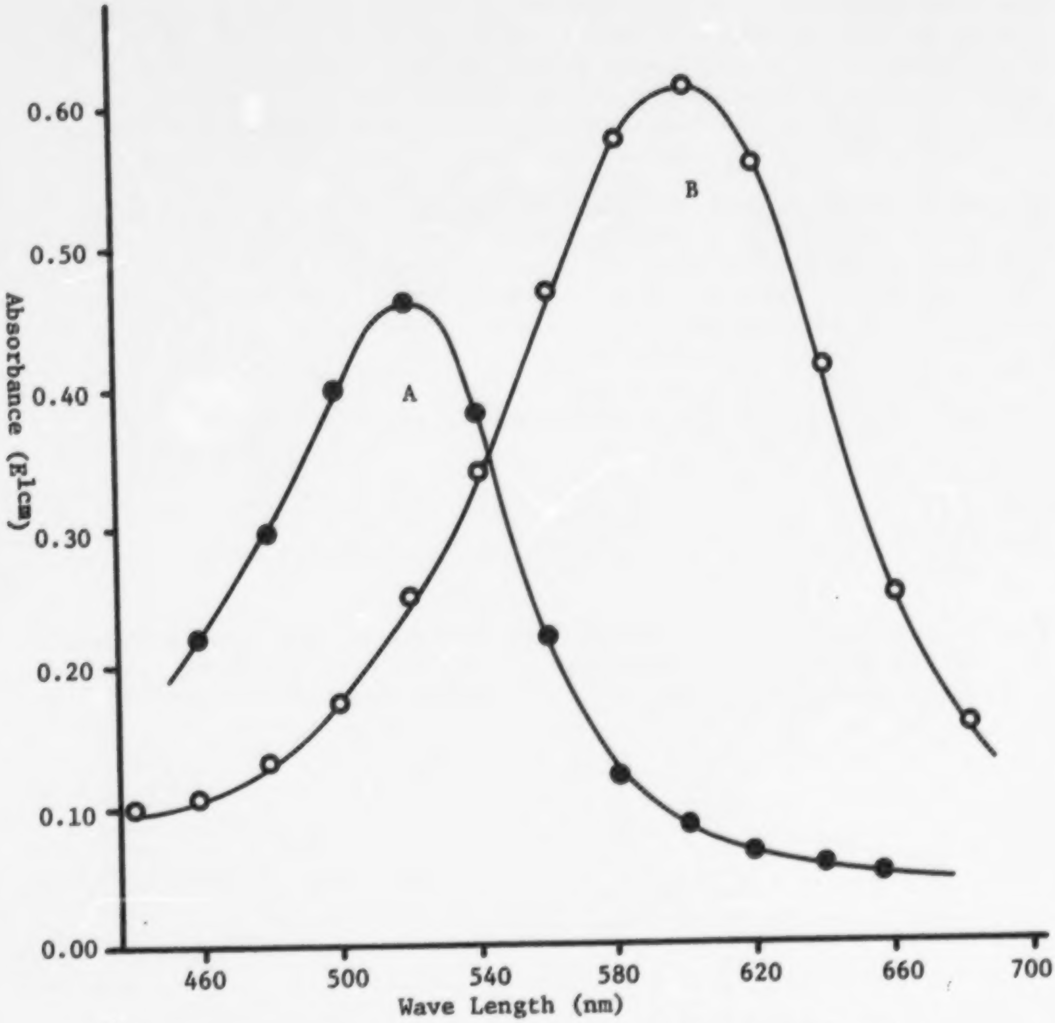


FIGURE 31. ABSORPTION SPECTRA OF THE 2,4-DINITROPHENYLAZO--DERIVATIVE OF 1-(N-NAPHTHYL)-ETHYLENEDIAMINE

A: In acidic solution; water 8.00 ml, acetic acid 0.50 ml and concentrated HCl 0.50 ml. 50 μg as coprostanol equivalent in 10 ml of the solution λ_{max} = 520 nm

B: In basic solution; pyridine-water. 45 μg as coprostanol equivalent in 10 ml of the solution. λ_{max} = 600 nm

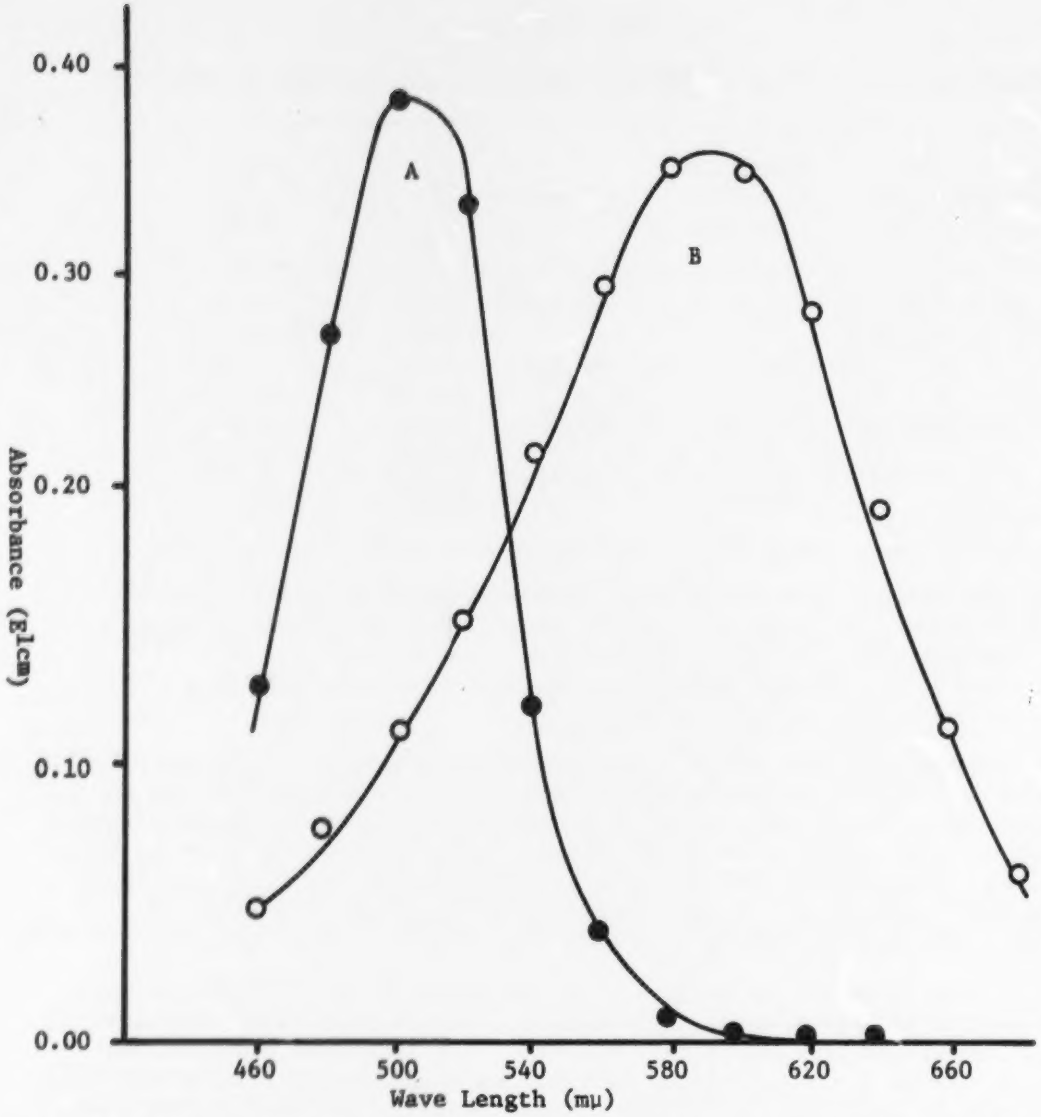


FIGURE 32. ABSORPTION SPECTRA OF 2,4-DINITROPHENYLAZO--DERIVATIVE OF α-NAPHTHYLAMINE

A: In acidic solution; water 8.00 ml, acetic acid 0.50 ml, concentrated HCl 0.50 ml. 25 μg as coprostanol equivalent in 10 ml of the solution. λ_{max} = nm

B: In basic solution; pyridine-water. 20 μg as coprostanol equivalent in 10 ml of the solution. λ_{max} = 590 nm

TABLE 28

ABSORPTION INTENSITIES OF THE AZO COMPOUNDS AND REAGENT BACKGROUNDS

Coupling Agent	Condition of Color Solution*	$E°\lambda$ max**	$E^{10}_{\mu g}$ $(E_{\mu g})$	E^{10}_{rb} (E^{10}_{rb})
1-(N-naphthyl)-ethylenediamine	acidic	32,700	0.008 (0.025)	---- (-----)
	basic	56,900	0.015 (0.050)	0.009 (0.030)
α-naphthylamine	acidic	53,200	0.014 (0.046)	0.001 (0.004)
	basic	57,200	0.015 (0.051)	0.013 (-----)

* The solvent system, acetic acid-concentrated HCl-water (9:0.5:0.5) was used for acidic conditions and pyridine-water (variable ratios) for basic conditions.

** Molar extinction coefficient, as coprostanol equivalent.

The intense pink to orange color of the azo compound, 2,4-dinitrophenly-azo-α-naphthylamine-hydrochloride, was stable in acidic solution for at least 30 minutes, however, the color gradually faded and finally turned yellow. It was found that the fading of the color was not due to the effect of periodate, but probably to autoxidation, as shown in Figure 33, where the effect of time on the color intensity was examined. In Figure 34, the absorption intensity was calibrated by using pure 2,4-DNPH.

3. Calibration--Known amounts of coprostanol (0.25 to 50.00 μg) were converted to coprostanone, hydrozonated, and the resultant hydrazone was hydrolysed at 100°C for 10 minutes. The liberated 2,4-DNPH was converted to the azo compound by coupling with α-naphthylamine and the absorption intensity determined. Unfortunately, the calibration test resulted in high and random reagent backgrounds, which were due to the appearance of white turbidity from m-dinitrobenzene that was derived from decomposition products of the 2,4-DNPH reagent. m-Dinitrobenzene is a stable compound, insoluble in water but soluble in many organic solvents. The solutions in organic solvents do not show absorption in the visible wave length range. If the compound were solubilized in one of the above solvents, the turbidity could be eliminated. Reich, et.al., [69] experienced the same problem, which they overcame by dissolving the 2,4-DNPH of a keto-steroid in chloroform.

A brief study was conducted to evaluate the solvent extraction and to

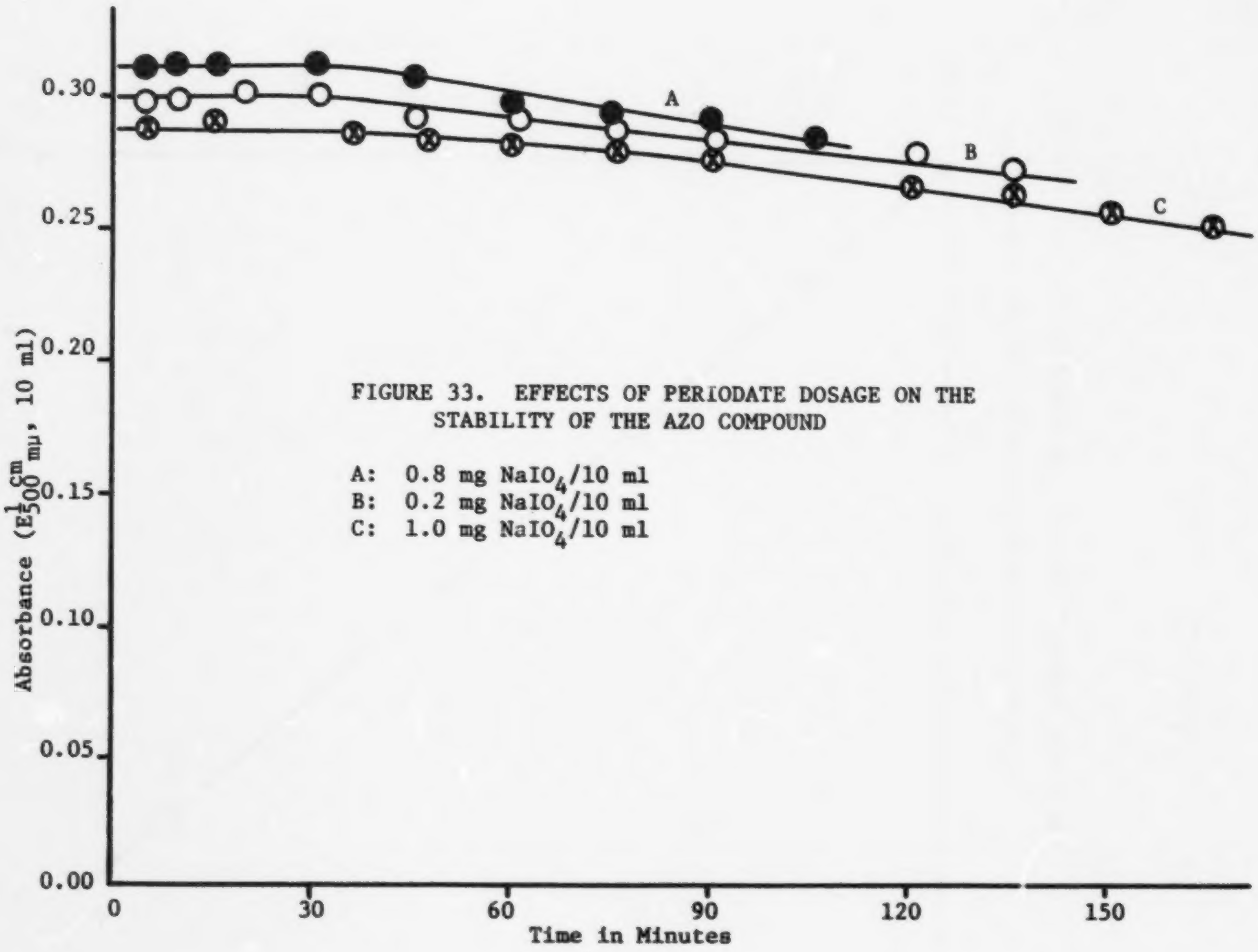


FIGURE 33. EFFECTS OF PERIODATE DOSAGE ON THE STABILITY OF THE AZO COMPOUND

A: 0.8 mg $NaIO_4$/10 ml
B: 0.2 mg $NaIO_4$/10 ml
C: 1.0 mg $NaIO_4$/10 ml

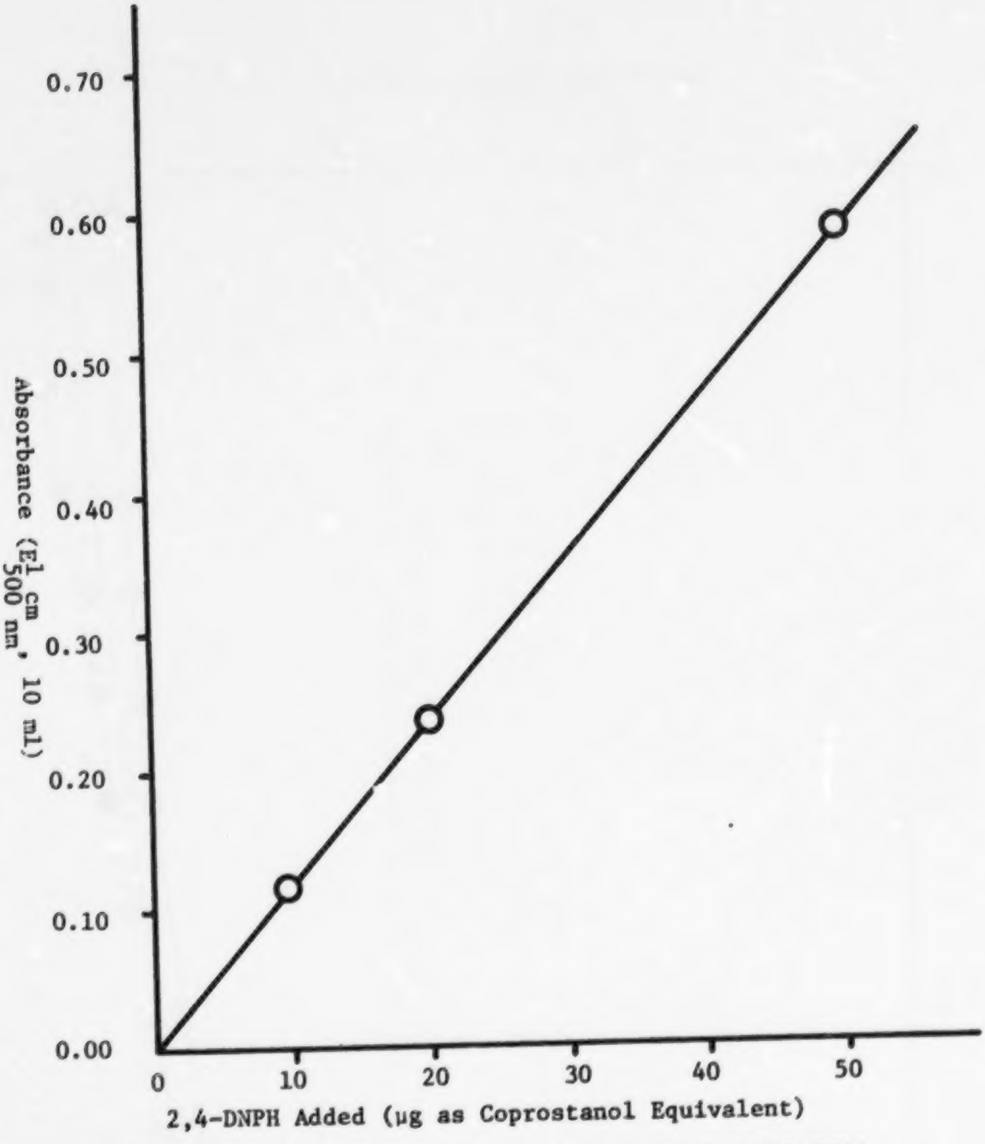


FIGURE 34. CALIBRATION OF 2,4-DINITROPHENYLHYDRAZINE

optimize the extraction conditions by neutralizing the acidic solution with pyridine.

In Table 29 the effects of the hydrolysis solvents and the pyridine base solutions are summarized. When the solution of the azo compound was not acidified by the hydrolysis solvent, there were no differences in the color intensity between the samples which were diluted with different strengths of the pyridine base solution. When a sample which was acidified with the hydrolysis solvent, was diluted with distilled water and extracted with ethylacetate, poor recovery of the azo compound resulted only 86 percent. The acidified samples, which were diluted with the pyridine base solutions, showed an improvement in recovery which indicated that the pyridine content was critical, i.e., when the solution of pyridine-acetic acid-water (10:8:82) was used to neutralize the acids, the recovery was still low--91 percent. If the pyridine content was increased to 15 percent pyridine, pyridine-acetic acid-water (15:5:80) 100 percent recovery was obtained. In the latter case, the reagent blanks became almost zero.

In summary, the optimum color development procedure was as follows:

1. Hydrolysis of coprostanone-2,4-DNPH--Dissolved first in 0.5 ml of glacial acetic acid, then added 0.5 ml of concentrated hydrochloric acid, and heated to 100°C in a boiling water bath for ten minutes.

2. Diazonium salts formation--The above solution was diluted with 5 ml of distilled water and one ml of periodate solution added, shaken thoroughly, and allowed to stand for about one minute.

3. Coupling reaction--To the above mixture, one ml of α-naphthylamine solution was added, shaken well and allowed to stand for a minute.

4. Extraction of the azo compound--The solution was then diluted with 5 ml of pyridine-acetic acid-water (15:5:80, by volume) and extracted twice with ethylacetate (3.0 ml first, 2.0 ml second). The ethylacetate extracts were poured into a 10 ml flask containing 3.00 ml of glacial acetic acid.

5. Stabilization of the color and measurement--To the flask, 1.00 ml of concentrated hydrochloric acid was added and the sample diluted with pure ethylacetate exactly to 10.00 ml. The color intensity was measured at 500 nm in a 1 cm cell.

Using the above procedure, 0.25 to 50.00 μg samples of coprostanol were used to prepare a calibration curve: The free coprostanol was converted to coprostanone and the resultant coprostanone was separated by an alumina column. The coprostanone was hydrozonated and the excess 2,4-DNPH reagent was removed at 80°C for 10 minutes. The calibration of coprostanol, 3.0 to 50.0 μg range, is shown in Figure 35 and the calibration, 0.25 to 3.00 μg, range, is shown in the Figure 36.

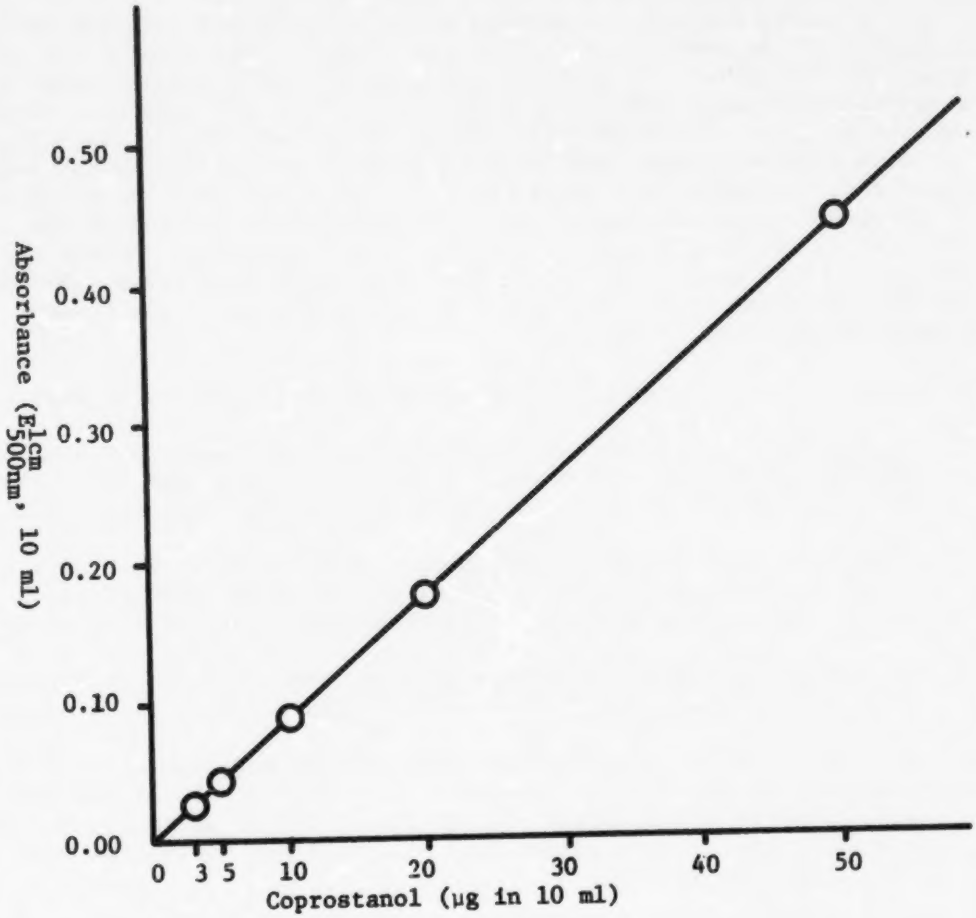


FIGURE 35. CALIBRATION OF COPROSTANOL (3-50 μg/Range)

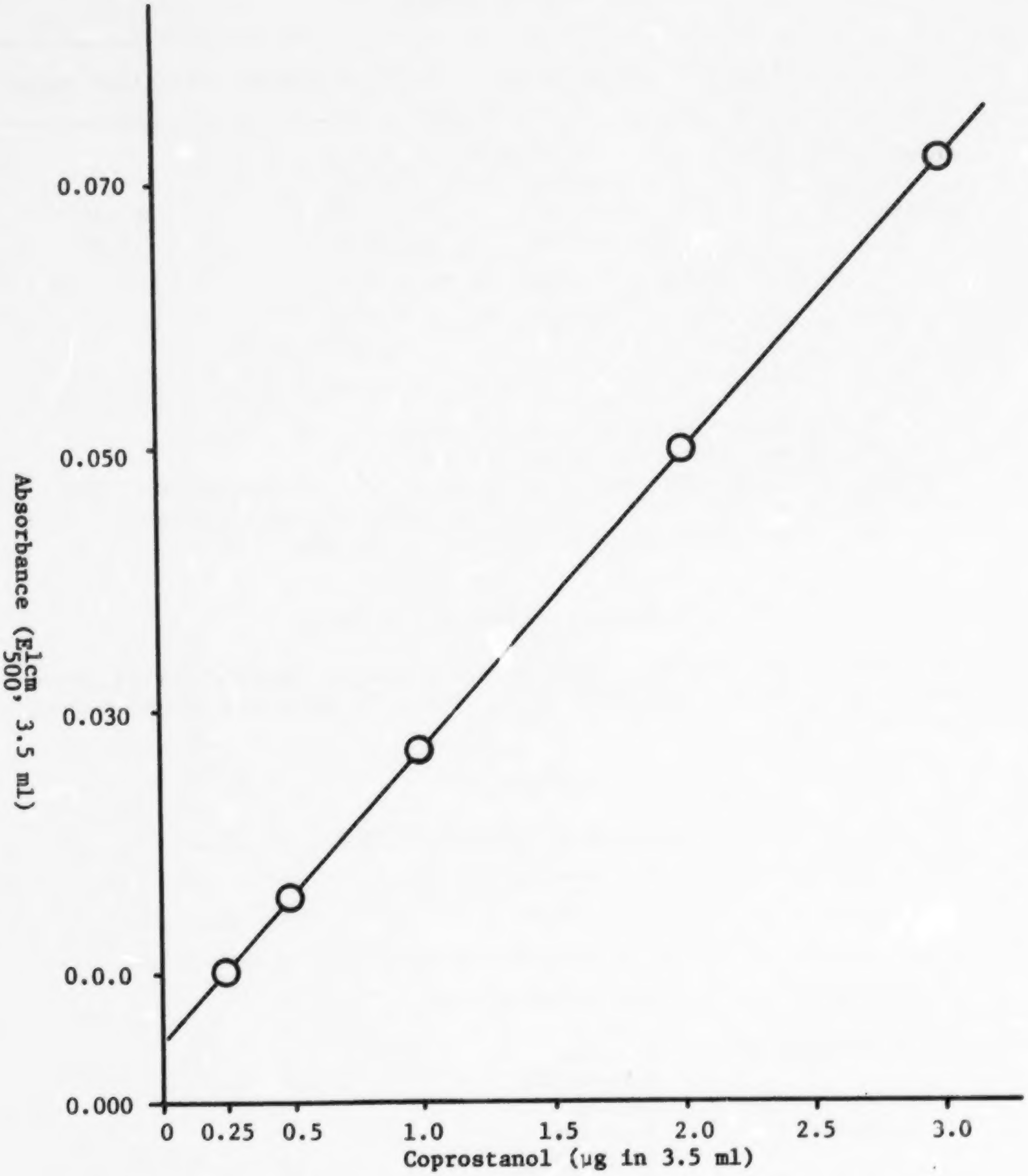


FIGURE 36. CALIBRATION OF COPROSTANOL (0.25-3 µg Range)

TABLE 29

EFFECT OF PYRIDINE BASE SOLUTION ON THE RECOVERY OF THE AZO COMPOUND BY ETHYLACETATE EXTRACTION

Hydrolysis Solvent*	10% Pyridine**	15% Pyridine***	Distilled Water
Absent	0.513**** (100%)	0.514 (100%)	
Present	0.465 (91%)	0.516 (100%)	0.442 (86%)
	Reagent Blank	0.000 (0.000 - 0.003)	

* Pyridine-acetic acid-water (10:8:82).
** Pyridine-acetic acid-water (15:5:80).
*** 0.5 ml of glacial acetic acid and 0.5 ml of concentrated HCl.
**** Absorbence in 10 ml of ethylacetate-acetic acid-concentrated HCl (6:3:1) mixed solvent, at 500 mμ in one cm cell.

Evaluation of Analytical Time

The total analytical time required for a single sample was estimated roughly to be two hours and a half. Table 30 shows the time required for each step.

TABLE 30

ESTIMATE OF ANALYTICAL TIME

Process	Step	Time (Minutes)	
1. Recovery	Hexane Extraction	20	
	Drying	10	30
2. Saponification	Boiling	5	
	Extraction	10	
	Drying	5	20
3. Oxidation	Reaction	20	
	Extraction	10	30
4. Chromatography	Hexane Elution	15	
	5% Ethylacetate Elution	5	
	Drying	5	25

TABLE 30

ESTIMATE OF ANALYTICAL TIME (Cont.)

Process	Step	Time (Minutes)	
5. Hydrazonation	Hydrazone Formation	5	
	Removal of the Reagent	5	
	Extraction	10	
	Drying	5	25
6. Color Development	From the Hydrolysis to the Azo dye formation	15	
	Extraction	5	
	Color Measurement	5	25
	Total Analytical Time		2:35

The above time must be considered a minimum or an attainable goal when performed by a well-trained analyst. However, the time can be reduced by modifying the procedure. For example, if the saponification of the hexane extracts in Step 2 is combined with the oxidation of the extracts, the analytical time will be reduced by 15 minutes by eliminating the extraction and drying steps in the saponification process. If the elution rate is increased in the chromatographic separation of coprostanone, the time will be reduced, and so on. Several attempts to reduce the analytical time were studied.

Summary of Results

It was found that the saponification and oxidation steps could be combined by increasing the amount of chromic acid slightly and the elution time could be reduced by 10 minutes; an overall reduction of about 25 minutes.

1. Reduction of Analytical Time by Combination of Saponification and Oxidation: The feasibility of the combination was evaluated. Cholesterol was included in order to estimate the background derived from 100 μg of cholesterol. The results are shown in Figure 37. Cholesterol and coprostanol were oxidized in about ten seconds and the resultant coprostanone represented 100 percent recovery in ten minutes, and the first oxidation product of cholesterol, Δ^5-cholesten-3-one, disappeared completely after ten minutes, as shown in Figure 37 where the recovery of coprostanone and Δ^5-cholesten-3-one are plotted with time. The results obtained here were almost the same as those of the uncombined oxidation except that additonal chromic acid of the Sarett

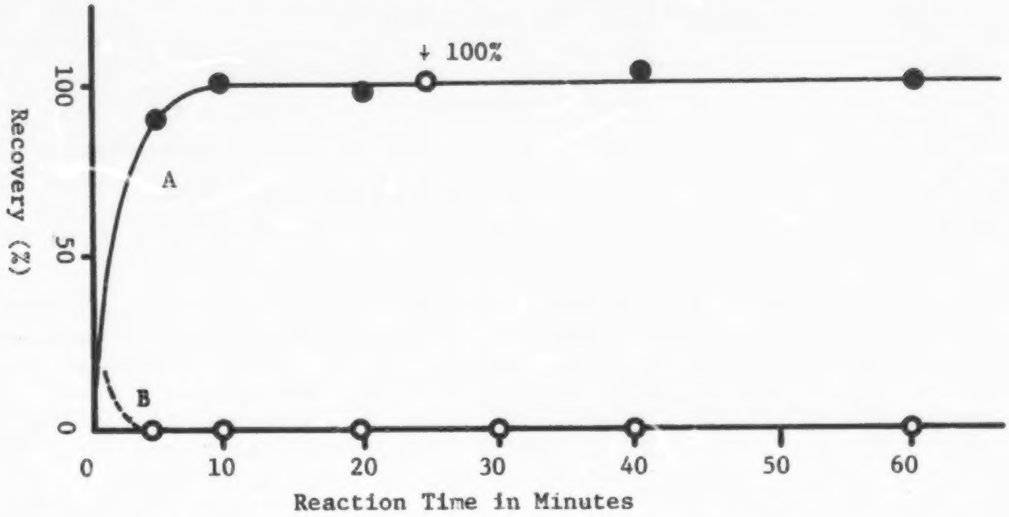


FIGURE 37. RECOVERY OF COPROSTANONE BY THE COMBINED PROCEDURE OF SAPONIFICATION AND OXIDATION

A. Coprostanone
B. Δ^5-Cholesten-3-one

reagent was consumed. The dosage of chromic acid-pyridine complex is critical in the combined procedure. It was found that 40 mg of chromic acid was sufficient for successful analysis. It was found, further, that the effect of the oxidation products of cholesterol or the color background was negligible, as shown in Table 31.

TABLE 31

COLOR BACKGROUND OF OXIDATION PRODUCTS OF CHOLESTEROL

Time of Oxidation (minutes)	Recovery Based on GLC Peak Height (inches)		Color Intensity (E^{10}_{rb})
	Cholesterol	First Oxidation Product	
5	0.00	0.07	0.010
10	"	0.00	0.000
20	"	"	0.004
30	"	"	0.001
40	"	"	0.001
60	"	"	0.002

2. Rapid Chromatographic Separation: The time for the chromatographic separation of coprostanone on an alumina column was 25 minutes as shown in Table 30; 15 minutes for dissolving the sample in hexane and a hexane wash, 5 minutes for elution and 5 minutes for drying the eluate. The column used was a funnel tube, 3.5 mm in diameter, packed with 0.5 grams of 80 to 200 mesh alumina. The elution rate of this column was 0.6 ml per minute. A study was conducted to determine whether the separation of coprostanone could be effectively accomplished when the elution rate was increased to 1.5 ml per minute by using a larger diameter column. In Table 32, the recovery of coprostanone and cholesterol are shown. In the first 5 ml of the 5 percent ethylacetate elution, coprostanone was recovered in 100 percent yield. The time for the separation was 15 minutes, 10 minutes for the elutions of the hexane extracts, washing with hexane and 5 percent ethylacetate, and 5 minutes for drying. This procedure reduced the analytical time by 10 minutes without losing efficiency. These changes could reduce the overall analytical time to just over two hours.

This 2N H_2SO_4 solution does not contain 10 percent $NaSO_4$. Sodium acetate in the reaction mixture forms soduim sulfate by reacting with 2N H_2SO_4, which is sufficient to effect salting out, and if the 2N H_2SO_4 contains 10 percent Na_2SO_4, it causes low recovery of coprostanone by precipitating soduim sulfate crystals when the coprostanone is extracted with hexane.

TABLE 32

SEPARATION OF COPROSTANONE AND CHOLESTEROL ON AN ALUMINA COLUMN

Solvent	Elution Volum (ml)	Recovery % Coprostanone	Cholesterol
Extracting Hexane	6	0.0	0.0
Washing Hexane	5.0	0.0	0.0
5% Ethylacetate	1.0	0.0	0.0
	1.0	39.3	0.0
	1.0	56.0	0.0
	2.0	4.7	0.0
	5.0	0.0	0.0
Ethylacetate	5.0	0.0	100.0

Summary of Colorimetric Method

The following is a summary of the analytical method:

1. Recovery of Coprostanol: Place one liter of the sample water in a 2-liter separatory funnel, and add 3 ml of concentrated sulfuric acid and 40 grams of sodium chloride crystals. The sample is extracted with 50 ml of hexane by shaking for two minutes. Transfer the water layer to another 2-liter separatory funnel and extract with another 50 ml of hexane. Discard the water layer and combine the two hexane layers. The hexane is washed with 25 ml of 70 percent ethanol twice and 25 ml of acetonitrile saturated with hexane twice. The hexane layer is evaporated to reduce the volume to a few milliliters under vacuum on a water bath at a temperature below 60°C. The concentrated solution is then transferred to a test tube with a transfer pipet and dried.

2. Saponification and Oxidation: The dried residues are dissolved in 1.0 ml of 0.5N NaOH in absolute methanol and heated to boiling until the volume is reduced to 0.2 ml or less, cooled and dried. The dried residues are dissolved in 0.5 ml of glacial acetic acid, 1.0 ml of the Sarett reagent added, warmed for 20 minutes at 50° ± 1°C in a water bath, and then cooled to room temperature. The mixture is diluted with 10 ml of 2N H_2SO_4 and extracted with 2 ml of hexane three times.

3. Separation of Coprostanone: The hexane solution is passed through an alumina column, and the column is washed with 5 ml of pure hexane. The coprostanone fraction is obtained from the column by eluting with 5 ml of 5 percent ethylacetate in hexane, and the eluate dried.

4. Hydrazonation: The well-dried 5 percent ethylacetate eluate is dissolved in 0.5 ml of carbonyl-free methanol, 0.5 ml of 2,4-DNPH reagent added and allowed to stand for 5 minutes. The methanol solution diluted with 5 ml of 2N H_2SO_4 in carbonyl-free water and 1.0 ml of potassimum dichromate solution is added. The mixture is then heated at 80°C for 5 minutes in a water bath, shaken several times and cooled to room temperature. The solution is diluted with 5 ml of 4N NaOH and extracted with 2 ml of hexane three times. The hexane is evaporated to dryness by blowing air over the surface.

5. Color Development: The well-dried, hydrazonated coprostanone is dissolved in 0.5 ml of glacial acetic acid and 0.5 ml concentrated hydrochloric acid added. The mixture is heated for 10 minutes in boiling water (100°C) cooled in cold water and then diluted with 5 ml of distilled water. To the diluted solution, 1.0 ml of sodium periodate solution is added and shaken well. Then 1.0 ml of α-naphthylamine solution is added, shaken thoroughly and allowed to stand 5 minutes. The solution is then diluted with 5 ml of the pyridine base solution and extracted with pure ethylacetate twice, first 3 ml and second 2 ml. The ethylacetate is stored in a 10 ml flask containing 3.00 ml of glacial acetic acid, to which 1.00 ml of concentrated hydrochloric acid is added. The mixture solution is diluted with pure ethylacetate to exactly 10.00 ml. The pink color of the azo compound is measured at 500 nm in a 1 cm cell using a spectrophotometer.

Application of the Colorimetric Method

The colorimetric method was applied to actual field samples and the results obtained are discussed in this section.

The colorimetric method used to quantify coprostanol and its analogs is based upon changing them to coprostanone and then eliminating interfering materials such as cholesterol, hydrocarbons, esters and other hexane-soluble constituents which are more polar than coprostanone. Sewage and natural waters contain many organic compounds; for example, about 50 mg of hexane extractables were present in one liter of raw sewage, the structures of most of which have not been elucidated. These could interfere with a colorimetric method, since many of these constituents have chemical natures similar to coprostanol (alcohol) or coprostanone (ketone).

To establish the colorimetric method as a measure of fecal contamination and domestic wastewater pollution, the method has to detect only compounds of fecal origin. The optimum method would detect only coprostanol. A field trip was conducted to estimate the interference of unknown constituents in the sample waters on the colorimetric determination of coprostanol.

Sampling stations that were included were:

1. Orlando City Sewage Treatment (West and East Plants): Raw sewage,

trickling filter, activated sludge effluents and finished waters. Fifty to 300 ml samples were diluted with distilled water to one liter.

2. University of Florida Campus Treatment Plant: Raw sewage, 200 ml was diluted with distilled water to one liter. One liter of finished water was collected.

3. Lake waters: (a) Newnan's Lake (located east of the City of Gainesville) - One liter of the water was collected at the west shore boat harbor. The lake is characterized by high color concentration; (b) Lake Alice (located on the University of Florida campus) - One liter of the water was sampled at the outfall. The lake is eutrophied.

4. Tap water: City of Gainesville. One liter sample.

5. Control: Carbonyl-free water, one liter sample.

The coprostanol concentrations were determined both by GLC and by the colorimetric method as detailed in the summary. Results of the two methods are compared in Table 33.

Analyses of the lake samples by the colorimetric method detected small amounts of color-sensitive materials as coprostanol equivalent, which were not coprostanol. The GLC method did not detect any trace of coprostanol in these samples. Tap water and carbonyl-free water samples did not show any traces of the color-sensitive materials. This would suggest that the lake waters gave the natural background of the colorimetric method. Since these lakes are characterized as organic rich waters, this should lead to maximum natural background interference.

As expected for the sewage samples, the colorimetric method gave higher results than the GLC method. The coprostanol contents measured by the colorimetric method are plotted versus those of GLC measurement in Figure 38. The regression coefficient was computed to be 1.61 and the standard error of the ratios of the colorimetric to the GLC results was calculated to be 0.34.

The above results indicate that sewage samples contained some colorimetry-sensitive materials. This overestimation should not lead to the conclusion that the colorimetric method is not suitable for the evaluation of domestic waste pollution, as discussed below.

The gas chromatograms of the coprostanol fractions (5 percent ethylacetate fraction) of sewage samples showed the existence of an unknown compound between the two oxidation products of cholesterol resulting from oxidation with the Sarett reagent, as shown in Figure 39. The following calculations were conducted to infer statistically the contribution of the unidentified peak compound versus the heights of the coprostanone peaks. The correlation coefficient was computed between

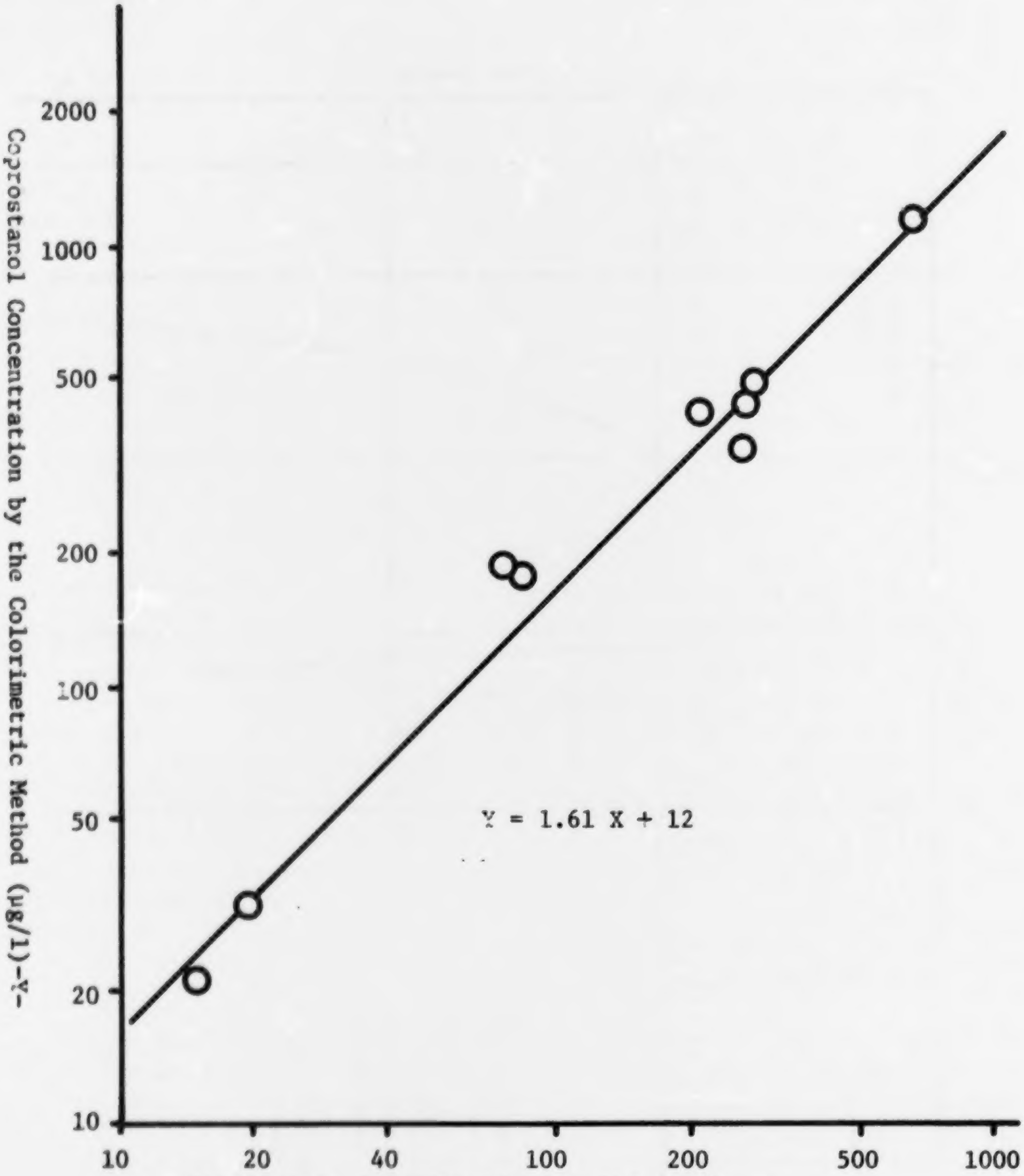


FIGURE 38. COMPARISON OF THE COLORIMETRIC METHOD VALUES AND ON GLC VALUES

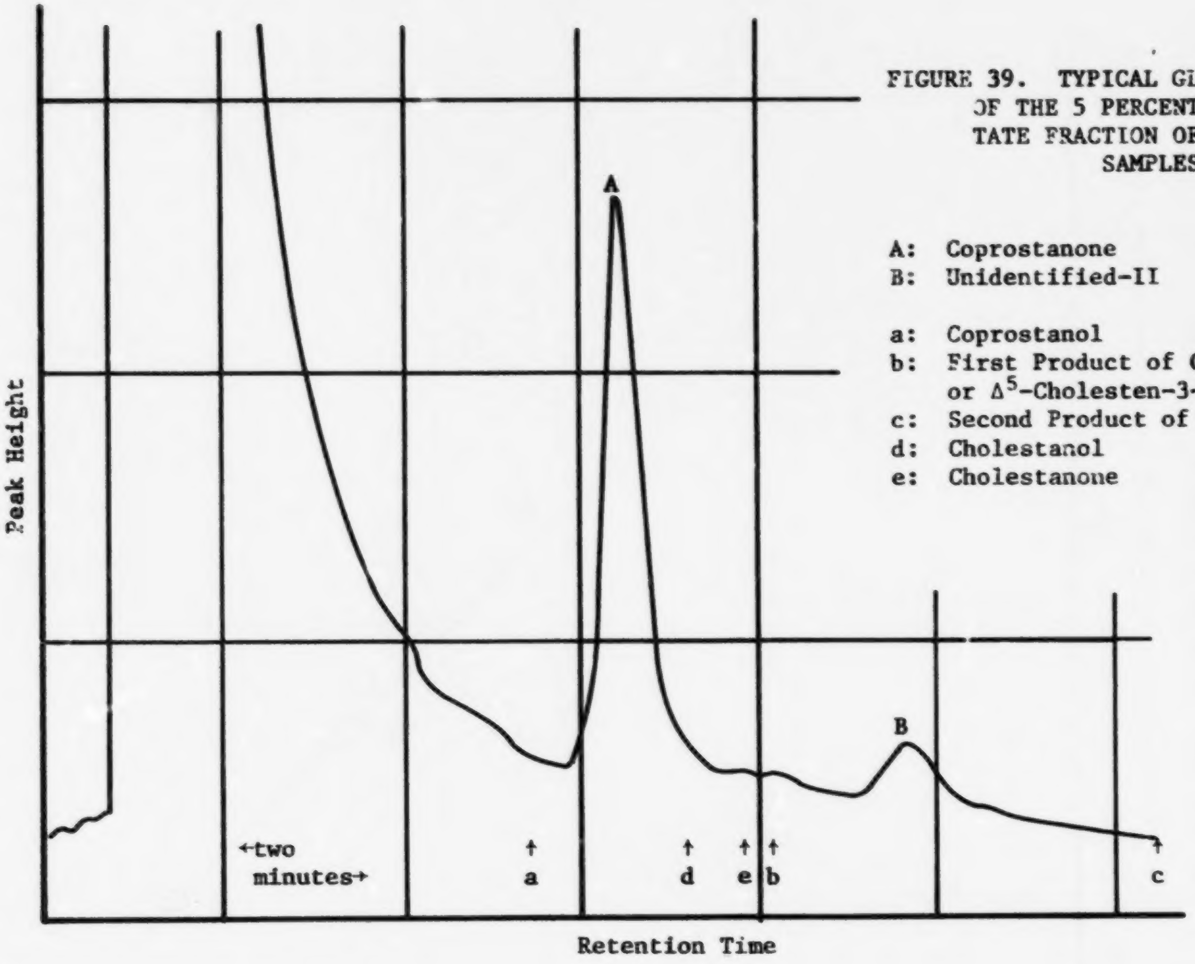


FIGURE 39. TYPICAL GLC PATTERN OF THE 5 PERCENT ETHYLACETATE FRACTION OF SEWAGE SAMPLES.

A: Coprostanone
B: Unidentified-II

a: Coprostanol
b: First Product of Cholesterol or Δ^5-Cholesten-3-one
c: Second Product of Cholesterol
d: Cholestanol
e: Cholestanone

the two values. The standard deviations:

$$S_x^2 = (0.375)^2 \; ; \qquad S_Y^2 = (0.0240)^2$$

The correlation coefficient:

$$r = 0.58$$

The confidence level of the value r was computed by using the variable $z = 1/2 \ln \frac{1+r}{1-r}$ with standard deviation approximately 1/N-3. The following range of the correlation coefficient ρ was obtained at the 5 percent level of significance:

$$-0.14 < \rho < 0.90$$

As the value obtained ($r = 0.58$) was between the above values, the hypothetical coefficient is accepted with 95 percent confidence. The above statistical inference indicates that the unidentified compound might have contributed to the overestimation of coprostanol as determined by the colorimetric method. Since the correlation coefficient was not large, there may be other factors which caused the higher results in the colorimetry.

TABLE 33

COPROSTANOL CONCENTRATION OF FIELD SAMPLES

Sample			Coprostanol Concentration		Ratio
			GLC (μg/l)	Colorimetry	Color./GLC
Sewage Plant I	1.	Finished	19.8	31.7	1.601
	2.	Trickling Filter	76.7	186	2.425
	3.	Activated Sludge	218	409	1.877
	4.	Raw Sewage	292	494	1.692
Sewage Plant II	5.	Finished	272	343	1.261
	6.	Trickling Filter	285	439	1.540
	7.	Raw Sewage	680	1,146	1.685
Sewage Plant III	8.	Finished	15.1	21.1	1.397
	9.	Raw Sewage	88	169	1.920
Lakes	10.	Newnan's Lake	u.d.*	2.5	
	11.	Lake Alice	u.d.*	2.5	
Control	12.	Tap Water	u.d.*		
	13.	Carbonyl-Free Water	(0.0)	0.0	

* Undetected by GLC, lower than 0.05 μg/l (shown as 0.022 in Table 11 for Lake Alice).

I. Orlando City Sewage Treatment Plant (West).

II. Orlando City Sewage Treatment Plant (East).

III. University of Florida, Campus Treatment Plant (Weeken aples).

TABLE 34

STATISTICS OF THE OVERESTIMATION OF COPROSTANOL BY COLORIMETRIC METHOD

Sample Number (from Table 33)	Ratio of Overestimation (X)	Ratio of Unidentified Peak (Y=b/a)	Peak Height (Inches)	
			Coprostanone (a)	Unidentified (b)
1	0.601	0.109	1.01	0.11
2	1.425	0.178	1.01	0.18
3	0.877	0.154	2.80	0.48
4	0.692	0.119	7.65	0.91
5	0.261	0.113	3.90	0.44
6	0.540	0.117	2.73	0.32
7	0.685	0.111	3.96	0.44
8	0.397	0.130	3.22	0.42
9	0.920	0.109	3.76	0.41

$\Sigma X = 6.398$ $\Sigma X^2 = 5.472776$ $\Sigma XY = 0.857163$
$\Sigma Y = 1.104$ $\Sigma Y^2 = 0.149002$

An estimate of the neutral steroid levels in a domestic sewer system would give an approximation which can explain the higher results of the colorimetric method.

Danielsson and Tchen [74] reported that human excretion of neutral steroids was 500 to 700 mg per capita per day. If it is assumed that the average is 600 mg and use of water is 400 liters per capita per day, the concentration of neutral steroids in raw sewage is computed to be $\frac{600}{400} = 1.50$ mg/l. The concentrations of cholesterol and coprostanol in raw sewage that were obtained in this study were approximately 600 µg/l each. When these two sterols are subtracted from the neutral steroids, the concentration of other neutral steroids is calculated to be 1.50 - (0.60 + 0.60) = 0.30 mg/l. Since the coprostanol concentrations, as determined by the colorimetric method, did not include cholesterol, the total neutral steroids are 0.30 + 0.60 = 0.90 mg/l, and the ratio of this concentration to the coprostanol concentration is determined to be 0.90/0.60 = 1.50. This is almost the same ratio as the regression coefficient, which was calculated to be 1.61 from Figure 38. It can be concluded from the calculations, even with the approximations that were made, that some neutral steroids from human intestinal tracts might have contributed to the higher results of the colorimetric determination.

The chemical nature of the unidentified compound present in the gas chromatograms of sewage samples was examined by the following experiment: Two hundred milliliters of raw sewage was placed in a 2-liter separatory funnel and diluted with 800 ml of distilled water, then 3 ml of concentrated H_2SO_4 was added, followed by 40 grams of NaCl, which was dissolved. The mixture was extracted with hexane and the hexane was washed with 70 percent ethanol and acetonitrile and dried in a test tube. The dried residues were redissolved in 4.00 ml of hexane and 1.00 ml was pipeted into three tubes and dried. They were then treated as follows:

Tube A. Not treated.
Tube B. Saponified.
Tube C. Saponified and oxidized.

The dried residues, after the above treatments, were dissolved in 6 ml of hexane and passed through alumina columns. The columns were eluted first with 5 ml of pure hexane, then 5 ml of 5 percent ethylacetate in hexane and then 5 ml of pure ethylacetate. The eluates were evaporated to dryness. The residues from each fraction were gas chromatographed. The results are shown in Figure 40.

The unidentified compound (Unidentified-II) appeared in the 5 percent ethylacetate fraction (coprostanone fraction) only when the sample was saponified and oxidized (tube C). The ethylacetate fraction (sterol fraction) of unoxidized samples (tubes A and B) showed the existence of another unknown peak (unidentified I) with a retention time shorter than that of Unidentified II. The Unidentified I was not observed in the 5 percent ethylacetate fraction of the oxidized sample (tube C).

The above evidence indicates that the unknown compound which appeared in the coprostanone fraction and seemed to have caused higher results by the colorimetric method, was a ketone formed by oxidation with the Sarett reagent. It might have been a keto-sterol, i.e., an alcohol, before oxidation. That the unknown compound might have been present in sewage in the form of a keto-sterol, was suggested by the behavior of the second oxidation product of cholesterol, 6β-hydroxy-cholesten-3-one, which is a keto-sterol and appeared in the sterol fraction from the alumina column. Correspondingly, it is possible that the unknown compound (Unidentified II) was a polyketo-compound.

On the other hand, an experiment was performed where the hexane extracts of sewage samples were saponified, esterified, and then oxidized, both with chromic acid anhydride in acetic acid and with the Remieu-von Rudloff reagent (periodate with catalytic amount of permanganate in approximately 80 percent acetic acid). The gas chromatograms of the hexane fraction (ester fraction) from an alumina column indicated the existence of an unidentified peak (Unidentified IV) as shown in Figure 41 (cholesterylacetate was oxidized completely without coprostanylacetate being affected). The compound, Unidentified IV, was not found to be the oxidation product of cholesterylacetate, and therefore, might have been derived from sewage constituents. The oxidation products of cho-

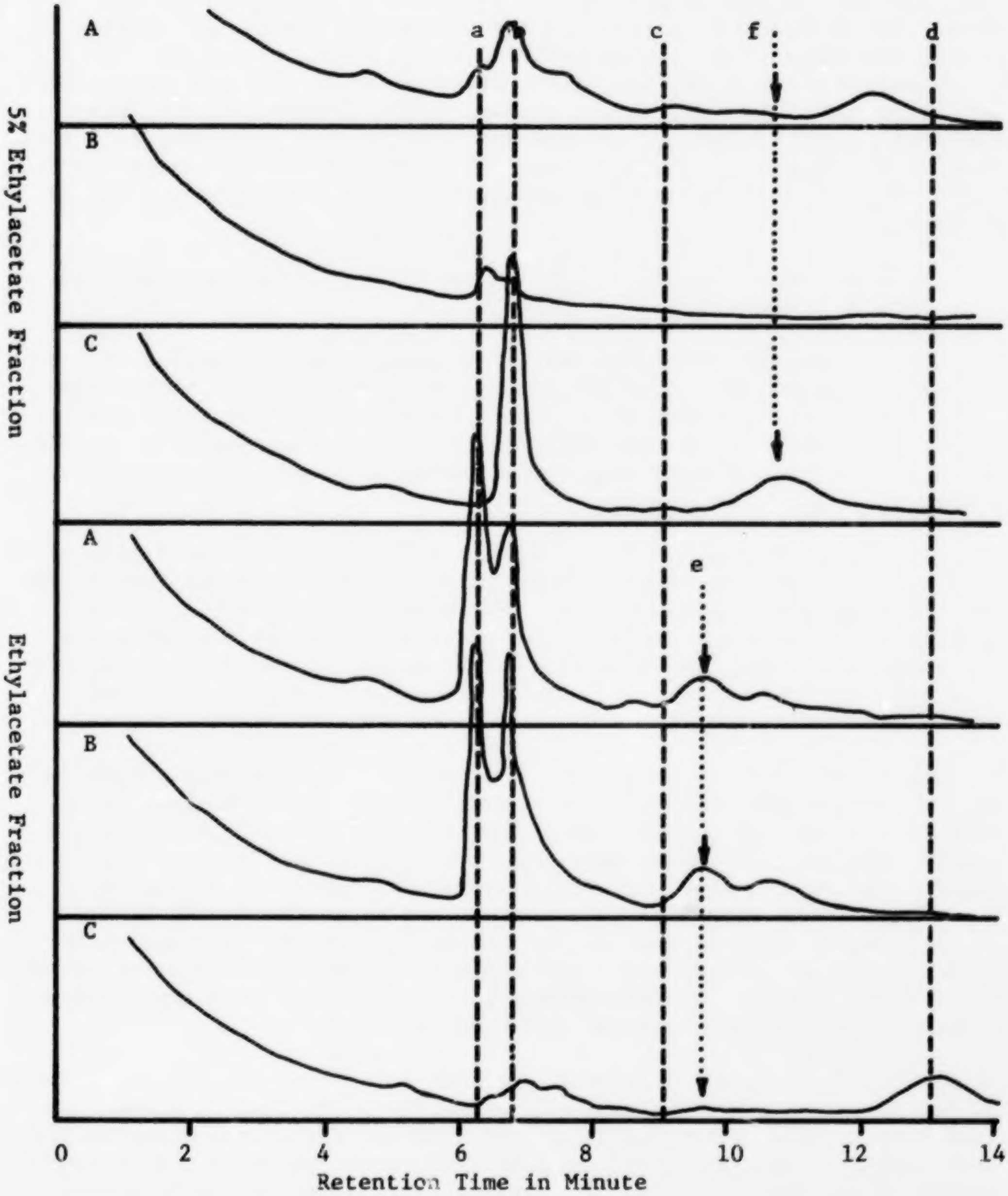


FIGURE 40. GAS CHROMATOGRAMS OF THE 5% ETHYLACETATE AND ETHYLACETATE FRACTIONS FROM AN ALUMINA COLUMN

A: Not Treated
B: Saponified
C: Saponified and Oxidized

a: Coprostanol
b: Cholesterol or Coprostanone
c: Δ^5-Cholesten-3-one
d: Second Oxidation Product of Cholesterol

e: Unidentified-I
f: Unidentified-II

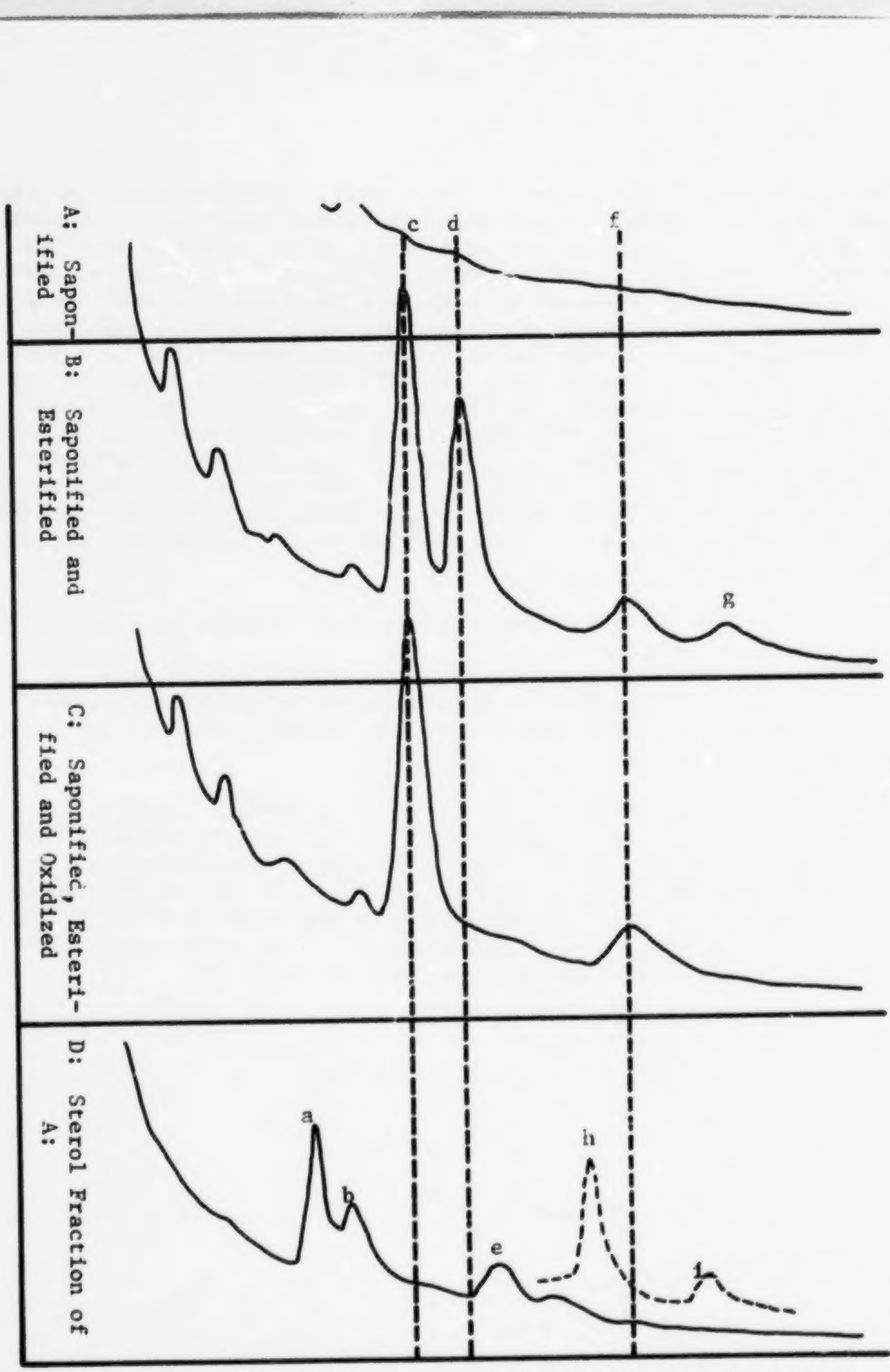


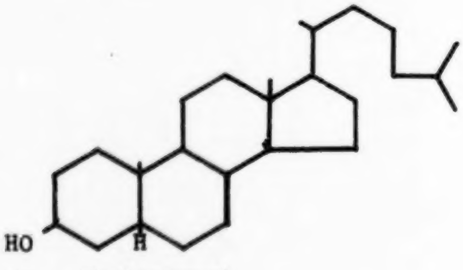
FIGURE 41. GAS CHROMATOGRAMS OF HEXANE FRACTION OF SEWAGE SAMPLES
a: Coprostanol; b: Cholesterol; c: Coprostanyl acetate; d: Cholesteryl acetate; e: Unidentified-III; f: Unidentified-IV; g: Unidentified-V; h-i: Oxidation products of cholesteryl acetate (not eluted with hexane).

lesterylacetate were eliminated by the alumina chromatography. As the Remieu-von Rudloff reagent cleaves double bonds, Unidentified IV seems to be a saturated compound. Although there is not a direct proof to show that Unidentified IV and Unidentified II have the identical precursor, the behavior of these two compounds on alumina columns and their reactivities to the saponification and oxidation reagents, suggested that the Unidentified IV was the ester and the Unidentified II was the ketonic form of an identical compound. The peak at the detention time of 5.6 minutes of the saponified (but not esterified) sample, disappeared after it was esterified and two new peaks appeared, one of which was decomposed by the oxidation and another was unchanged (Unidentified II), was the same as the evidence that was obtained in the other experiment, i.e., Unidentified I appeared in the saponified (but not oxidized) sample and it disappeared by oxidation with the formation of a new peak (Unidentified II).

The above evidence indicated that Unidentified II might have been a saturated polyketonic compound.

Cholestanol and cholic acid--the possibilities of cholestanol (IX) and cholic acid (X) as contributors to the overestimation by the colorimetric method, were considered.

1. Cholestanol (5α-cholestan-3β-ol): Fecal materials contain, in addition to the major neutral steroids (cholesterol and coprostanol) such other neutral steroids as cholestanol, 7-dehydro-cholesterol, lathosterol, cholestanone and coprostanone [74,75]. The gas-chromatographic nature of cholestanol was determined and it was found that the Unidentified II was not cholestanone. Cholestanone was not detected in the final fraction of sewage samples, as shown in Figure 39.



IX. Cholestanol

2. Cholic Acid: The acid is a major constituent of human bile acids. The fecal excretion of bile acids has been reported to be 800 mg per day per person [76]. The amount is significantly large compared to neutral steroids excretion of 500 to 700 mg per day per person [74]. An experiment was conducted to determine if the acid would affect the

colorimetry. Two hundred μg of cholic acid was treated by saponification, oxidation and alumina column separation. The result showed that cholic acid was not detected by the colorimetric method. The acid was not eluted by 5 percent ethylacetate in hexane from an alumina column even after it formed a ketone structure, because it still possessed polar carboxyl groups in the side chain. If the carboxyl groups were split off by microbial action to form 20 or 17-ketones, the products would have contributed to the colorimetric overestimation. Although this degradation process is not confirmed, the cleavage of the side chain would seem to be possible, forming 17-ketones, by analogy with the fact that progestrone metabolites were reported to have formed 17-ketones by splitting of the methoxy group at 17C by micro-organism [77].

It has not been possible to identify positively the exact structure of the substances that gave high values for the colorimetric coprostanol analyses but it is almost certain that they are of fecal or urinal orgin.

Summary of Field Studies

The colorimetric method was applied to field samples. The method overestimated the coprostanol concentrations of sewage samples by a factor of 1.6 as compared to the results obtained by GLC. Some natural waters gave a trace of colorimetry-sensitive materials which were not coprostanol. Tap water did not show any color sensitivity.

An unknown compound which seemed to have caused the overestimation of the colorimetry, appeared on the gas chromatograms of sewage samples. The column characteristics and the reactivities of the unknown compound to saponification, oxidation and esterification indicated that the unknown compound was a saturated keto-steroid from fecal or urinal excretion.

Although the colorimetric method measured not only coprostanol but also other materials, it can be used effectively for the evaluation of fecal and domestic wastewater pollution for the following reasons:

1. The color-sensitive materials seem to originate in fecal or urinal excretion, and
2. The natural background of the colorimetric method is low.

The colorimetric method is simple, rapid and sensitive. The results can be obtained within two hours, and the method is less expensive than GLC. It could be used for routine analyses by sewage treatment plant operators and field observers.

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APPENDIX

A. Standards

1. Coprostanol was obtained from Applied Science Laboratories, Inc. (State College, Pa.). 100.0 mg of coprostanol was dissolved in 100.0 ml of hexane and 2.00 ml pipetted into test tubes and dried. The working standard was prepared by dissolving the 2.00 mg of coprostanol in the tubes in 10.00 ml of hexane, 0.250 ml of which was taken in another test tube with a micro pipet and dried. The tubes contained 50.00 μg of coprostanol.

2. Cholesterol was obtained from K & K Laboratories, Inc. (Plainview, N. Y.). 100.0 mg was dissolved in 100.0 ml of absolute methanol (or ethanol) and 2.00 ml aliquots of the solution were pipetted into test tubes and dried. The working standards were prepared by dissolving 2.00 mg of cholesterol in 10.00 ml of hexane, 0.50 ml (or 0.250 ml) was measured into test tubes by micro pipets. Each tube contains 100.0 μg (or 50.0) of cholesterol.

3. Coprostanone, Δ^5-cholesten-3-one and cholestanol were obtained from K & K Laboratories, Inc. The hydrocarbons (n-docosane and n-octacosane) were obtained from Applied Science Laboratories, Inc.

B. Reagent

1. Sarett reagent: 20 grams of chromic acid anhydride (0.2 mol) was dissolved in the mixed solvent of 31.6 grams of pyridine and 150 ml of glacial acetic acid by adding the chromic acid slowly and by keeping the temperature below 20°C. The solution was diluted with glacial acetic acid to 250 ml. The reagent is photosensitive. It was stored in a brown bottle. The working solution was prepared by diluting the stock solution with an equal volume of glacial acetic acid and filtering with a glass fiber filter using vacuum. One milliliter of the working solution contained 40 mg of chromic trioxide (CrO_3).

2. Saponification reagent for coprostanol esters: 0.5N NaOH in absolute methanol. Two grams of sodium hydroxide was dissolved in 100 ml of absolute methanol (analytical grade) and stored in a glass bottle with a screw cap. It was kept out of contact with atmospheric carbon dioxide.

3. 2,4-DNPH reagent for the standard and hydrazonation of coprostanone: 0.1019 gram of 2,4-DNPH crystals was placed in a 50 ml beaker, dissolved in two milliliters of concentrated sulfuric acid and 3 ml of distilled water added. The mixture was diluted exactly to 200 ml with carbonyl-free methanol in a volumetric flask. One milliliter of the solution contained 1.000 μg of 2,4-DNPH (as coprostanol equivalent).

APPENDIX

4. 2,4-DNPH-sulfate reagent for preparation of carbonyl-free water and methanol: To 0.4 gram of 2,4-DNPH in a 50 ml beaker was added 2 ml of concentrated sulfuric acid. The 2,4-DNPH was dissolved by adding 3 ml of distilled water. The mixture was then diluted with 10 ml of absolute methanol.

5. Periodate solution for diazonium formation: The stock solution was prepared by dissolving 2.0 grams of sodium periodate in 100 ml of carbonyl-free water. 2.00 ml of the stock solution was diluted with 48 ml of the carbonyl-free water, for the working solution.

6. Coupling reagents: (a) α-naphthylamine solution--2.00 grams of α-naphthylaminehydrochloride was dissolved in one liter of distilled water to which 1 ml of concentrated hydrochloric acid was added. It was stored in a dark bottle. The working solution was prepared by diluting the stock solution by 20 fold with carbonyl-free water. 1.00 ml of the working solution was contained 100 μg of α-naphthylaminehydrochloride, which was equivalent to about 220 μg of coprostanol; (b) N-(1-naphthyl)-ethylenediamine solution--0.50 gram of the dihydrochloride was dissolved in 500 ml of distilled water. The solution was stored in a dark bottle.

C. Solvents and Solutions

1. Carbonyl-free water: One milliliter of concentrated sulfuric acid and 2 ml of 2,4-DNPH sulfate reagent were added to one liter of distilled water, allowed to stand overnight, and then distilled.

2. Carbonyl-free methanol: One milliliter of concentrated sulfuric acid and 2 ml of 2,4-DNPH-sulfate reagent were mixed with one liter of absolute methanol (analytical grade), refluxed for one hour and then distilled.

3. Hexane, ethanol and acetonitrile: Analytical grade.

4. Pyridine base solution: Pyridine-(sp. grade)-Glacial acetic acid-distilled water (15:5:80 by volume).

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